THE ESSENTIAL OIL FROM PEEL AND FLOWER OF *CITRUS MAXIMA*

By

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต

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The graduate school, Silpakorn University accepted thesis entitled “THE ESSENTIAL OIL FROM PEEL AND FLOWER OF *CITRUS MAXIMA*” by Napaporn Thavanapong in partial fulfillment of the requirements for the degree of master of pharmacy, program of pharmacognosy.

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เปลือกผลและดอกส้มโอ (*Citrus maxima* Merr.) พันธุ์ขาวใหญ่ จากอำเภอบางคนที จังหวัดสมุทรสงคราม ประเทศไทย ถูกนำมาเตรียมน้ำมันหอมระเหย โดยน้ำมันจากเปลือกผลส้มโอได้จากการเตรียมด้วยการบีบเย็น (CP) การกลั่นด้วยไอน้ำในสภาวะควบคุมความดัน (VP) และการสกัดด้วยคาร์บอนไดออกไซด์ในสภาวะของไหลยิ่งยวด (SCP) เมื่อวิเคราะห์หาองค์ประกอบทางเคมีของน้ำมันหอมระเหยด้วยแก๊สโครมาโตรกราฟฟีและแก๊สโครมาโตรกราฟี-แมสสเปกโตรมิเตอร์ ผ่านแคปปิลารี่คอลัมน์สองชนิดคือ DB-5 และ carbowax พบสารที่เป็นองค์ประกอบทางเคมีหลักในน้ำมันหอมระเหยทั้งสามชนิดคือ limonene, myrcene, β-pinene, α-pinene, sabinene, linalool เป็นต้น น้ำมันหอมระเหยดอกส้มโอสกัดด้วยคาร์บอนไดออกไซด์ในสภาวะของไหลยิ่งยวด (SC-f) พบ limonene ในปริมาณสูง แต่มีสารกลุ่มออกซิจิเนเทต คอมพาวด์โดยรวม ในปริมาณที่น้อยกว่าเมื่อเปรียบเทียบกับน้ำมันหอมระเหยดอกส้ม (neroli) ที่ได้จากการสกัดดอก *Citrus aurantium* var. *amara* และน้ำมันหอมระเหยดอกส้มโอที่ได้จากการกลั่นด้วยไอน้ำ จากรายงานก่อนหน้านี้ น้ำมันหอมระเหย SCP และ SC-f สามารถยับยั้งการเจริญเติบโตของเชื้อ *S. aureus* ที่ความเข้มข้นต่ำสุด (MIC) เท่ากับ 2.5 µl/ml ในขณะที่ neroli สามารถยับยั้งได้ 50% แต่ CP และ VP ไม่มีฤทธิ์ที่ความเข้มข้นเดียวกัน น้ำมันหอมระเหยทุกตัวอย่างไม่สามารถยับยั้ง *E. coli* ในขณะที่ SCP, SC-f และ neroli ยับยั้ง *C. albican* ได้ 50% ที่ความเข้มข้น 2.5 µl/ml

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The peel and flower of *Citrus maxima* Merr. cultivar khao–yai were collected from Bangkontee District, Samutsongkarm Province of Thailand. The peel oils of *C. maxima* were prepared by cold pressing (CP), vacuum steam distillation (VP) and supercritical carbon dioxide extraction (SCP) methods. The major compounds that were detected in SCP, CP and VP essential oils by GC and GC-MS analysis using DB-5 and carbowax capillary column were limonene, myrcene, β-pinene, α-pinene, sabinene, linalool etc. *C. maxima* flower which was extracted by supercritical carbon dioxide extraction (SC-f) showed higher content of limonene, but less total oxygenated compounds than commercial neroli derived from *Citrus aurantium* var. *amara* and steam distillation *C. maxima* flower oil from the previous reports. SCP and SC-f showed antimicrobial activity against *S. aureus* at the same minimum inhibitory concentration (MIC) equaled to 2.5 µl/ml. Neroli showed 50% inhibition for *S. aureus* at 2.5 µl/ml whereas CP and VP did not active. All of the oil samples could not inhibit the growth of *E. coli*, while SCP, SC-f and neroli at the concentration of 2.5 µl/ml presented 50% inhibition against *C. albican*.

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Student's signature ……..……..…

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LIST OF ABBREVIATIONS

$[n]_D$	Refractive index
$[\alpha]_D$	Optical rotation
$^{\circ}C$	Degree celsius
µl	Micro litter
Abs	Absorbances
amu	Atomic mass unit
BP	British Pharmacopoeia
Carbowax	polyethylene glycol capillary column
CFU	Colony forming unit
cm	Centimeter
CO_2	Carbon dioxide
CP	Essential oil derived from *Citrus maxima* peel, extracted by CPM
CPM	Cold pressing method
DB-5	5% phenyl & 95% dimethylpolysiloxane capillary column
DMSO	Dimethyl sulfoxide
EI	Electron impact
etc.	et cetera
EtOH	Ethanol
FID	Flame ionized detector
GC	Gas chromatography
GC-MS	Gas chromtography-Mass spectroscopy
gm	Gram
i.e.	Example
IR	Infrared spectrum
kg	Kilogram
KI	Kovats Index
MHB	Mueller Hinton broth

LIST OF ABBREVIATIONS (CONTINUED)

MIC	Minimum inhibitory concentration
min	Minute
ml	Millimeter
MPa	Mega pascal
N/S	Not state
neroli	Essential oil derived from *Citrus aurantium* var. *amara*, extracted by organic solvent, from Italy (commercial product)
ng	Nano gram
nm	Nano meter
OV-1	100% dimethylpolysiloxane capillary column
ppm	Part per million
Psi	Pound per square inch
r^2	Correlation coefficient
RI	Retention index
rpm	round per minute
RSD	Relative standard deviation
R_t	Retention time
SCF	Essential oil derived from *Citrus maxima* flower, extracted by SCO_2
SCO_2	Supercritical carbon dioxide extraction
SCP	Essential oil derived from *Citrus maxima* peel, extracted by SCO_2
SD	Standard deviation
SDA	Sabouraud dextrose agar
spp.	Species
TSA	Tryptic soy agar
TSB	Tryptic soy broth

LIST OF ABBREVIATIONS (CONTINUED)

TTHD	Thai Traditional and Herbal Development Center
USA	United State of America
v/w	Volume by weight
VP	Essential oil derived from *Citrus maxima* peel, extracted by VSD
VSD	Vacuum steam distillation

CHAPTER I
INTRODUCTION

The pomelo (*Citrus maxima* Merr.), a native plant of Asia, which is best cultivated in China, southern Japan, Vietnam, Malaysia, Indonesia and Thailand. The fruit of pomelo is commonly eaten fresh or made as juice. It is also popular for jam and syrup. In traditional medicine, the fruit peel has been used for cough, swellings, and epilepsy, because of the effectiveness of the volatiles.[1] The middle layer (albedo) of fruit peel is extracted for pectin which is used as dietary fiber for reducing body weight.[2]

The Citrus species are famous for the source of essential oils. *C. maxima* is the same as other Citrus species that contains essential oil glands in their fruit peel and flower petals. The Citrus peel oils have a strong and desirable aroma with refreshing effect. They have been used as flavoring in foods, beverages and pharmaceutical products. They also have been used as fragrance in perfumes, cosmetic and aromatherapy. The Citrus flower oils have the relaxing and hormone balancing effects which have been used in aromatherapy and perfumery.[3, 4]

In Thailand, there are lots of cultivar of *C. maxima* such as khao-num-puang, khao-poung and khao-yai which are popular as fresh eating. The *C. maxima* is peeled for their fresh pulp and juice that are sold both in Thailand and foreign countries. The high demand of pomelo in the market causes around one metric ton of the peel left as by products in each day. These wastes could be served as raw materials for essential oil industries. So the study of *C. maxima* peel essential oil could increase the value of the useless waste.

The extraction methods of Citrus essential oil are important, since these oils are very sensitive to heat. The old classical method used only for Citrus peel extract is cold pressing method. The smell of cold press oil is naturally, but contains a lot of impurities. The second classical method for essential oil extraction is steam distillation. This method is universal for most volatile oil extraction. It is an easy

method and gives pure essential oil separated from water. However, this method is used high temperature which some chemicals in essential oil may be volatile, especially Citrus oils. Some chemicals of Citrus oils are changed their structure by heat. Therefore the steam distillation is not the suitable method for Citrus essential oil extraction. Citrus essential oil extraction needs low temperature method which gives low impurities contamination. At present, supercritical carbon dioxide extraction is appropriate to solve the problems of the classical methods. It could extract essential oils with low temperature and no solvent used resulting in the improvement of the extract efficiency and the reduction of impurities.

Neroli is commonly oil used from Citrus species. This oil is produced from bitter orange flower (*Citrus aurantium* L.) by distillation or solvent extraction. It had been used in the high quality eau de cologne since the seventeenth century. High quality perfume and aromatherapy are always adding neroli in their recipes. Neroli has the relaxant effect with the balancing condition. It could cause restorative effect on the brain and stimulate the upper digestive organs. It also effectively controls bacterial and microbacterial infection, especially gastroenteritis and lung tuberculosis. In the perfume industry, neroli oil is widely used in blends with most other floral oils and absolutes, including jasmine oil, lavender oil, rose oil and its absolute, ylang-ylang oil, vanilla, as well as petigrain oil, bergamot oil, lemon oil, lime oil, grapefruit oil, and etc.[5] *C. maxima* flower have a similar scent as bitter orange flower. The *C. maxima* flower are bigger than bitter orange flower in size, and they also contain more oil glands on their petals. So, Citrus flower could give higher amount of essential oil than the bitter orange flower.[6]

Nowadays, there are no commercial *C. maxima* essential oil available. Because the extraction of flower essential oils needs modern methodology for reducing solvent residue, impurities and chemicals transformation and increasing yield. These factors influence on the oil quality. Therefore, supercritical carbon dioxide extraction is considerable advantage over the other extraction methods.

It is true that the gardener grows *C. maxima* trees for their fruits not flower. However, after fertilization of *C. maxima* flower, their petals always fall down and they are allowed to wither with no use. Then, the development of *C. maxima* flower

oil extraction is profitable for the gardener. This may help to increase gardener's income and to support the perfumery industries in Thailand. Because of *C. maxima* is unique in Southeast Asia and its flower oil could generally substitute neroli that import from western countries.

The aims of this study were to compare the essential oil from different extraction methods of peel and flower of *C. maxima* that could be applied to industries. The comparison were done by differentiate and comparable the chemical constituents in the oils from different extraction methods and sources. In this study, the *C. maxima* peel oils were pressed by cold pressing, vacuum steam distillation and supercritical carbon dioxide extraction methods. The *C. maxima* flower oil was obtained from supercritical carbon dioxide extraction and compared to commercial neroli that was obtained from the solvent extraction method.

Goal and objective of this study

1. For study and comparison the chemical constituents of essential oil derived from *Citrus maxima* peel by the cold pressing, vacuum steam distillation and supercritical carbon dioxide extraction.

2. For study and comparison the chemical constituents of essential oil derived from *Citrus maxima* flower by supercritical carbon dioxide extraction and the commercial neroli derived from *Citrus aurantium* var. *amara* that was found in Thailand market.

3. For study antimicrobial activities of *C. maxima* peel oil, and flower oil compare to that of neroli.

CHAPTER II
LITERATURE REVIEW

1. *Citrus maxima*

Citrus maxima (pomelo or shaddock), family Rutaceae, is one of the famous fruits in Thailand. The other scientific or synonyms of pomelo are *Aurantium maximum* Burm. ex Rumph, *Citrus aurantium* L. var *grandis* L., *Citrus decumana* L., *Citrus grandis* Osbeck and *Citrus pamplemos* Risso. Pomelo is a indigenous plant of Malayu Island and the east of India. It is widespread in China, Japan, the Philippines, Indonesia, the United State of America and Thailand.[7,8] It can be cultivated in lots of provinces of Thailand. Its different local names from different parts of Thailand are Ma-O (North), Sung-Ou (Maehongson) and Som-O (Central).[9, 10, 11]

In Thailand, *C. maxima* was developed to plenty of cultivar for their pleasant taste and their names were depended on the cultivated areas. For example, khao-tong-dee, khao-num-puang, khao-hom, khao-poung, khao-pan and khao-yai varieties are found in the Central : Nakhon-Pathom, Samutsakhon, Samutsongkarm and Rachaburi provinces. While tang-gwar, ta-koy varieties are found in the North: Chainat, Nakhonsawan, Uthaithani, Phichit and Phitsanulok provinces. There were some varieties in the south such as hom-hadyai in Songkhla province.[7, 8]

C. maxima is medium sized tree. Its leaves have the small winged petioles. The flower are bisexual and smell sweet. The tree can flower when its age is four years old. The flowering in December – February is called Som-Pee when its produces lots of flower, and the flowering in August – September is called Som-Ta-Wai. The fruit is always round shape and big size. Fruit peel has the 3 layers: the outer layer is called flavedo or epicarp which has the oil glands, the medium layer is called albedo or mesocarp which is white in color and has plenty of spongy cells, and

the inner layer is called endocarb which is the edible portion of the fruit, the juice sags.[4, 8, 12]

C. maxima flower smell quite similarly to the *C. aurantium* var. *amara* flower (neroli). Neroli is the well known essential oil for aromatherapy and perfumery industry. The production of neroli starts to be considerable when the tree is about 10 years old, and reaches its maximum, when the tree is about 20-30 years old (5-25 kg of flower per tree per year). The harvest of the flower requires a considerable amount of work. It is time consuming, and causes of an increase in cost. The flower are picked at the time of their blooming under warm and sunny weather conditions. Those harvested in the early morning yield oil higher than those harvested in the afternoon. The closed blossoms yield lower, and give to the oil a green note. The collection of the flower should be selective, avoiding small leaves and petioles. The most common of extraction method for neroli producing are distillation and solvent extraction. Hydro- distillation, without the distilled or cohobated water recycle, at low pressure inside the alembic, for three hours could produce about 1.0 % average yield. Dipping the flower in water, prior to the distillation, increases the oil yield. The flower concrete is obtained by extraction with hexane or light petroleum with a yield that range between 0.2-0.3 % for the flower harvested in spring. The extractors used are the classical static type, with a load of 1,000-2,000 L, and the flower extracted immediately after the harvest could give higher yield than those stored for several hours. Moreover, dipping the flower in the solvent at different times gives a higher yield than one single extraction. From the concrete it is possible to obtain the absolute using methods for producing absolutes (extraction with alcohols, winterization, filtration and concentration). The yields are about 48-52% of concrete. The main components of neroli from distillation method were limonene, linalool, linalyl acetate, nerolidol, geranyl acetate, neryl acetate, geraniol, nerol, α-terpineol, methyl anthranilate and other aromatic compounds. The composition of neroli oils from Spanish and Tunisian which were obtained by hydro distillation and the oil extracted by supercritical carbon dioxide from bitter orange flower (*C. aurantium*) from Morocco showed the different contents between the two types of oils : monoterpene hydrocarbons and linalyl acetate (38.0 and 28.0%, 5.0 and 24.0% respectively).

Because of there were some chemical transformation of linalyl acetate during the distillation process, leading to the formation of monoterpene hydrocarbons and other monoterpenoids. Neroli from solvent extraction contained lower monoterpene hydrocarbons (5.9%) and higher linalyl acetate (16.8%) than the oil from distillation method.[4,13]

Chemical constituents of essential oil isolated from the flower of *C. maxima* in Vietnam by steam distillation was investigated. Limonene, linalool, nerolidol and farnesol are the main compounds.[60] However it has not been reported about the extraction of *C. maxima* flower by SCO_2.

2. Chemical constituents of *C. maxima*

There are plenty of reports about the chemical constituents in the plant parts of *C. maxima*. The reported compounds in this review have been searched until 25 November 2004 including alkaloids, amino acids, benzenoids, carbohydrates, carotenoids, coumarins, flavonoids, monoterpenes, sesquiterpenes, triterpenes and steroids (Table 1). Figure 1 shows the structures of monoterpenes and sesquiterpenes, which are found in *C. maxima* essential oil. The biological activities of *C. maxima* essential oil are shown in Table 2.

Table 1 Chemical constituents of *C. maxima*

Class	Compound	Plant part	Reference
Alkaloids			
	5-hydroxyacronycine	root bark, stem bark	14
	acriginine A	root	15
	atalafoline	stem bark	14
	baiyumine A	root bark	16, 17
	baiyumine B	root bark	16, 17
	buntanbismine	stem bark	18
	buntanine	root bark, stem bark	17
	buntanmine	stem bark	17
	citbismine A	root	19
	citbismine B	root	19
	citbismine C	root	19
	citbismine E	root	20
	citpressine I	root bark, stem bark	17, 21, 22
	citpressine I	root bark, stem bark	17, 22
	citracridone I	root bark, stem bark	17, 21, 22
	citracridone II	root bark	17, 21, 22
	citropone A	root bark	17, 23
	citropone B	root bark	17, 23
	citrusinine I	root bark	22
	geibalansine	stem bark	24
	glycocitrine I	root bark	17, 21, 22
	grandisine I	root bark	17, 21

Table 1 Chemical constituents of *C. maxima* (continued)

Class	Compound	Plant part	Reference
Alkaloids(continued)			
	grandisine II	root bark	17, 25
	grandisine III	root	25
	grandisinine	root bark, stem bark	17, 21, 22, 26
	honyumine	root bark, stem bark	21, 27
	natsucitrine II	root bark	21
	prenylcitpressine	root bark	17, 22
	preskimmianine	root bark	22
	pumiline	root	25
	caffeine	flower	28
Amino acids			
	alanine	leaf	29
	asparagine	leaf	29
	aspartic acid	leaf	29
	coline	leaf	30
	glutamic acid	leaf	29
	glycine	leaf	29
	proline	leaf	29
Benzenoids			
	crenulatin	root bark, stem bark	17
	diphenylamine	fruit juice, flavedo	31

Table 1 Chemical constituents of *C. maxima* (continued)

Class	Compound	Plant part	Reference
Benzenoids (continued)			
	n-methylanthranilate	leaf	32
	p-hydroquinone	root bark	17, 22
Carbohydrates			
	phytol	leaf	32
	synephrine	peel	33
	methyl antranilate	flower (essential oil)	34
	fructose	leaf	30
	glucose	leaf, fruit peel	30
	pectin	peel	35, 36, 37
Carotenoids			
	carotene	peel	38
	roseoside	peel	39
Coumarins			
	5-geranoxy-7-methoxy-coumarin	peel	40
	5-methoxy seselin	root bark, stem bark	21, 22
	5-methyltoddanol	stem bark	14
	6-hydroxy-methylherniarin	stem bark	24
	aurapten	peel	41, 42
	auraptene	peel	42
	bergamottin	peel	40

Table 1 Chemical constituents of *C. maxima* (continued)

Class	Compound	Plant part	Reference
Coumarins (continued)			
	bergapten	peel	43
	buntansin A	stem bark	14
	buntansin B	stem bark	24
	buntansin C	stem bark	24
	cedrelopsin	root bark	21
	(*Z*)-isokhellactone	stem bark	24
	citrubuntin	root bark, stem bark	14
	clausarin	root bark	17, 21, 22
	honyudisin	root bark	21
	isoimperatorin	peel	40, 44
	isomeranzin	peel	41, 43, 44
	meranzin	flavedo	43
	meranzin hydrate	flavedo	39, 43
	nordentatin	root bark	17, 21
	osthole	root bark	17
	pranferin	peel	41
	scopoletin	root bark, stem bark	21
	suberenone	stem bark	14
	suberosin	root bark	17
	thamnosin	root bark	21
	thamnosmonin	stem bark	24
	umbelliferone	root and stem bark, peel	17, 21, 41

Table 1 Chemical constituents of *C. maxima* (continued)

Class	Compound	Plant part	Reference
Coumarins (continued)			
	xanthotoxol	peel	41
	xanthoxyletin	root bark, stem bark	17, 21, 22
	xanthyletin	root bark, stem bark	17, 21, 22
Flavonoids			
	4’-5-7-8-tetramehoxy flavone	peel	45
	acacetin	leaf	46
	apigenin trimethyl ether	peel	45
	cosmosiin	leaf	30
	diosmetin	flavedo	47
	diosmin	flavedo, fruit juice	47
	eriocitrin	albedo	48
	hespeidin	peel, fruit juice	49
	honyucitrin	root bark	21
	isosinensetin	peel	45
	luteolin	fruit juice, peel, leaf	47
	naringenin	peel, leaf	50
	naringin	fruit juice, peel, leaf	50
	naringin glucoside	flavedo, albedo	48
	narirutin	fruit juice, peel, leaf	49
	neodiosmin	fruit juice, peel	47
	neoeriocitrin	fruit juice, peel, leaf	49

Table 1 Chemical constituents of *C. maxima* (continued)

Class	Compound	Plant part	Reference
Flavonoids (continued)			
	neohesperidin	fruit juice, peel, leaf	49
	neoponcirin	fruit juice, peel	47
	nobiletin	peel	45
	poncirin	albedo, leaf	47
	quercetin	fruit juice	51
	rhoifolin	fruit juice, peel, leaf	47
	rutin	peel, leaf	52
	sinensetin	fruit juice, peel	47
	tangeretin	peel	45
Monoterpenes			
	α-pinene	peel, flower, leaf	53
	α -terpineol	peel	54
	anethole	peel	55
	β-pinene	peel, leaf	17
	camphene	peel, flower	56
	camphor	flower	57
	citral	peel, flower, leaf	37, 58
	citronellal	leaf	58
	citronellol	peel	56
	farnesol	peel, flower	59, 60, 61
	geraniol	peel	56

Table 1 Chemical constituents of *C. maxima* (continued)

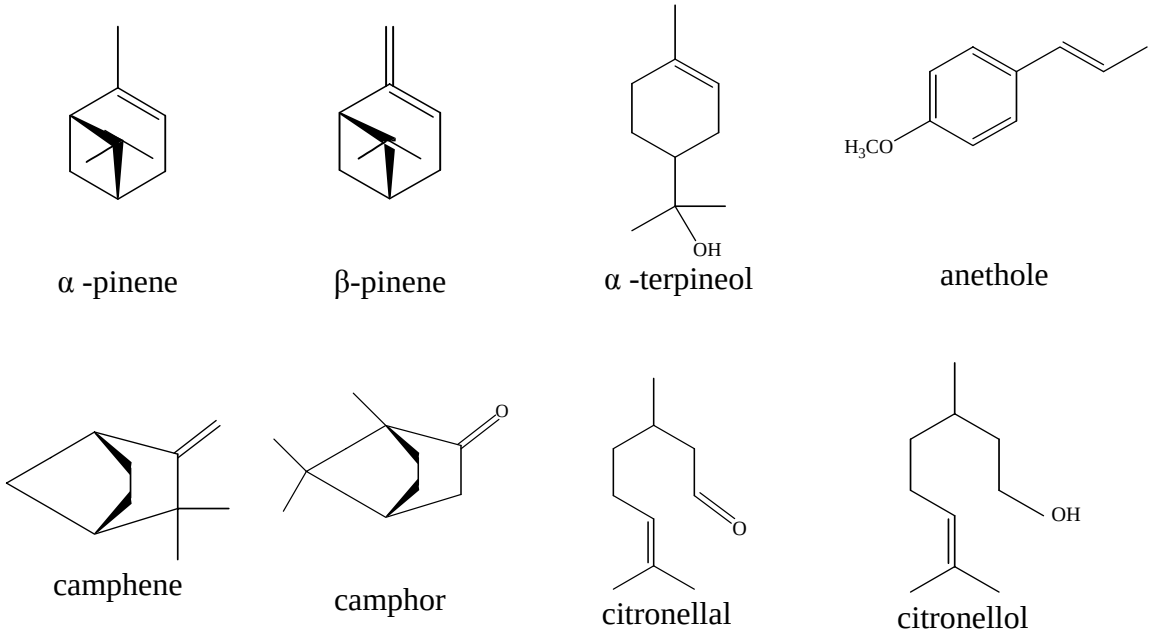
Class	Compound	Plant part	Reference
Monoterpenes (continued)			
	geraniol acetate	peel, leaf	58
	limonene	peel, flower, leaf	32, 58, 59, 60
	linalool	peel, flower, leaf	53, 55, 58, 59, 60, 62
	linalyl acetate	leaf	53, 58
	myrcene	leaf	56, 58, 59
	neral	peel, flower, leaf	58
	perilla aldehyde	peel	56
	terpinene	peel	56, 63
Sesquiterpenes			
	α -bisabolol	peel	61
	α -cadinene	peel	56
	α -copaene	peel	56
	α -cubebene	peel	54
	β-caryophyllene	peel, flower, leaf	32, 63
	β-copaene	peel	56
	β-cubenene	peel	56
	β-elemene	peel	56
	δ-cadinene	peel	56
	elemol	peel	56
	α -humelene	peel	32, 63
	nerolidol	peel, flower	56, 59, 60

Table 1 Chemical constituents of *C. maxima* (continued)

Class	Compound	Plant part	Reference
Sesquiterpenes (continued)			
	nootkatone	peel	54, 55, 56
	valencene	peel	56
Triterpenes			
	deacetynomilin	seed	64, 65
	deoxylimonin	seed, fruit pulp	66
	limonin	peel, leaf, seed, fruit pulp and juice	48
	nomilin	peel, leaf, seed, fruit pulp	67
	nomilin glucoside	peel	68
	nomilinic acid	seed	68
	obacunone	leaf, seed, fruit pulp	67, 64, 65, 68
	obacunone glucoside	seed	68
Storoids			
	β-sitosterol	peel, root	69
	campesterol	peel	69
	daucosterol	peel	70
	stigmasterol	root	71
Miscellaneous			
	α -tocopheral	peel	69
	ascorbic acid	fruit juice	69
	chlorophylls	peel	69

Table 1 Chemical constituents of *C. maxima* (continued)

Class	Compound	Plant part	Reference
Miscellaneous (continued)			
	decan-1-al	peel	56
	decyl acetate	peel	56
	dodecyl acetate	peel	56
	fumaric acid	leaf	72
	malonic acid	leaf	72
	nonan-1-al	peel	56
	nonyl acetate	peel	56
	octan-1-al	peel	56
	octyl acetate	peel	56
	oxalic acid	leaf	72
	succinic acid	leaf	72
	tartaric acid	leaf	72
	2-dodecenal	peel	61
	citric acid	fruit juice, peel	37
	decanoic acid	peel	61
	heptyl acetate	peel	61



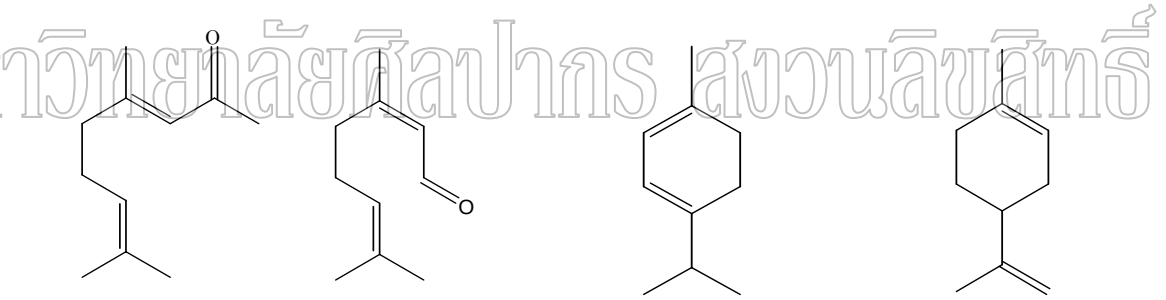
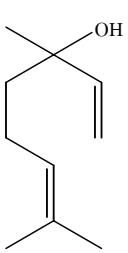
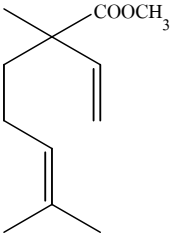


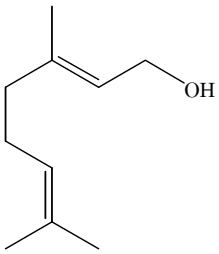
Figure 1 Monoterpenes and sesquiterpenes isolated from *C. maxima*



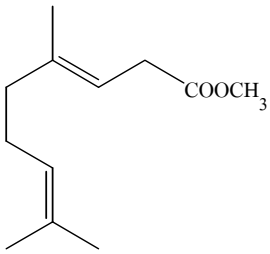
linalool



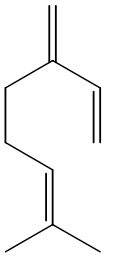
linalyl acetate



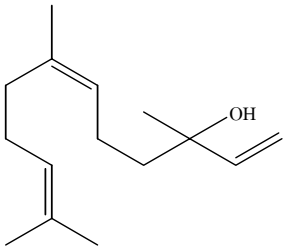
geraniol



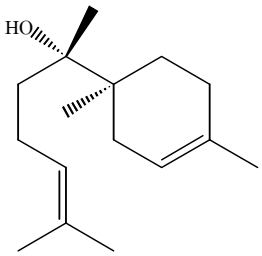
geranyl acetate



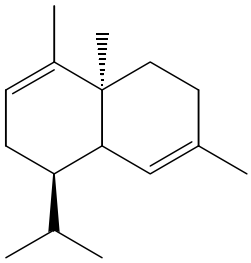
myrcene



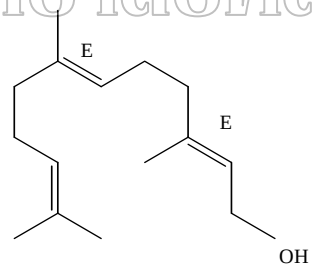
nerolidol



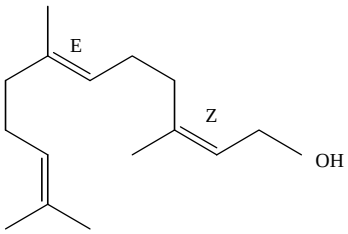
α -bisabolol



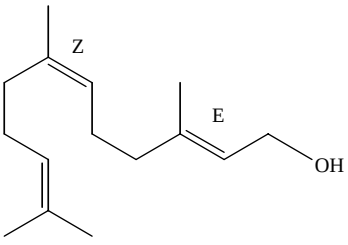
α -cadinene



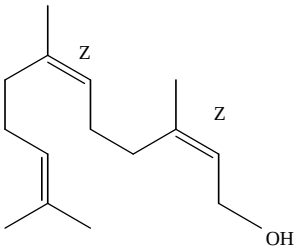
(*E*,*E*)-farnesol



(*Z*,*E*)-farnesol

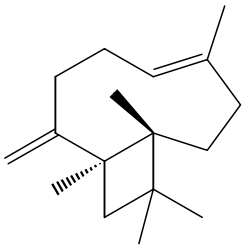


(*E*,*Z*)-farnesol

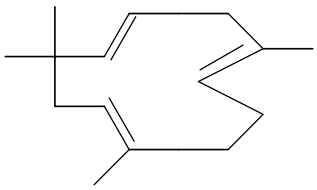


(*Z*,*Z*)-farnesol

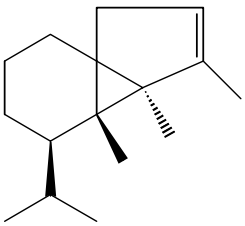
Figure 1 Monoterpenes and sesquiterpenes isolated from *C. maxima* (continued)



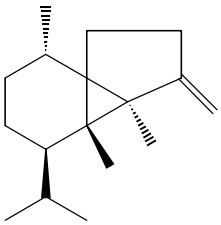
β-caryophyllene



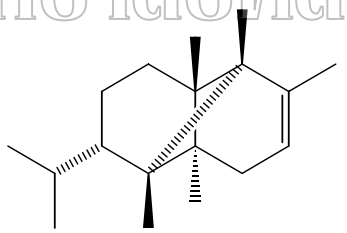
α -humulene



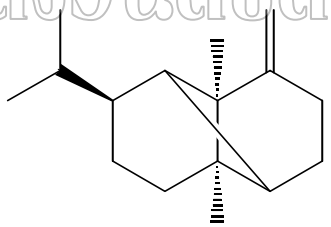
α -cubebene



β-cubebene

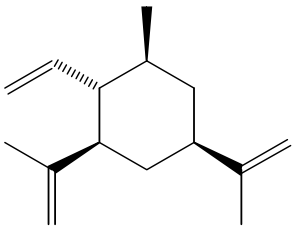


α -copaene

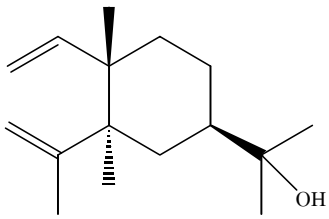


β-copaene

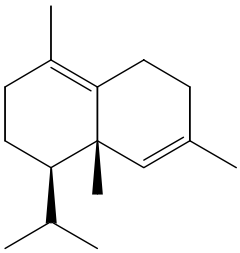
Figure 1 Monoterpenes and sesquiterpenes isolated from *C. maxima* (continued)



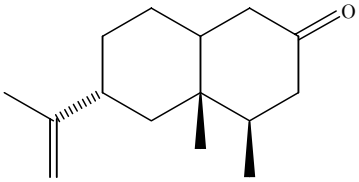
β-elemene



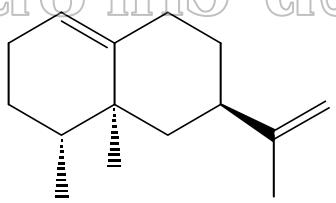
elemol



δ-cadinene



nootkatone



valencene

Figure 1 Monoterpenes and sesquiterpenes isolated from *C. maxima* (continued)

3. Biological activities of *C. maxima*

As they are the secondary metabolites, essential oils have been known as the protective compounds of plants. They have lots of bioactivities, especially antioxidant and anti-microbial activities.[86] Thus, they are used in skincare products for perfuming and aromatherapy purposes. There have been a plenty of researches of this property, since the anti-microbial has been the common activity found in essential oils from plants. The previous reports, until 25 November 2004, of *Citrus maxima* peel essential oil showed the anti-bacterial against *Staphylococcus aureus* and *Pseudomonas aeruginosa* with the concentration of 20.0 μl/agar disc[73], and the anti-fungal against *Trichophyton mentagrophytes* and *Microsporum audounil* with MIC 500 ppm. using agar plate method.[76, 77] There were no reports about activity of *C. maxima* flower essential oil yet.

Table 2 Biological activities of *C. maxima*

Acitvity	Part	Sample	Methods	Concentration	Result	Ref.
Antibacterial	Peel	Essential oil	Agar plates *Staphylococcus aureus* *Pseudomonas aeruginosa*	20.0 µl/agar disc	Active	73
	Peel	Essential oil	Agar plates *Esherichia coli* *Shigella dysenteriae* *Vibrio cholera*	20.0 µl/agar disc	Inactive	74
	Peel	EtOH extract	Agar plates *Esherichia coli* *Pseudomonas aeruginosa* *Staphylococcus aureus*	30.0 µl /agar disc	Inactive	75
Antifungal	Peel	Essential oil	Agar plates *Trichophyton mentagrophytes* *Microsporum audounil*	MIC 500.0 ppm	Active	76, 77
	Peel	Essential oil	Agar plates *Alternaria solan* *Curvularia luna* *Fusarium equiseti* *Macrophomina phaseolina*	N/S	Inactive	74

Table 2 Biological activities of *C. maxima* (continued)

Acitvity	Part	Sample	Methods	Concentration	Result	Ref.
Antiyeast	Peel	EtOH extract	Agar plates *Candida albicans*	30.0 µl /agar disc	Inactive	74
Antioxidant	Peel	Essential oil	DPPH	N/S	Active	42, 78
	Peel	70% EtOH extract	Inhibition of lipid peroxidase	N/S	Active	79
Larvicidal	Peel	Essential oil	Against mosquito larvae. *Culex quinquefasciatus* *Culex tritaeniorhynchus* *Aedes aegypti*	0.02 ml/L	Active	80
Smooth muscle relaxation	Peel	50% EtOH extract	Cell culture of intestine	250.0 µg/ml	Active	81
Uterine relaxation	Peel	50% EtOH extract	Cell culture of uterus (non-pregnant)	250.0 µg/ml	Active	81
Capillary permeability	Peel	50% EtOH extract	Cell culture	5.0 µg/ml	Increase	81
Inotropic effect	Peel	50% EtOH extract	Cell culture	1.0 µg/ml	Active	81

Table 2 Biological activities of *C. maxima* (continued)

Acitvity	Part	Sample	Methods	Concentration	Result	Ref.
Platelet aggregation inhibition	Peel	50% EtOH extract	Cell culture	5.0 µg/ml	Active	81

N/S : not state

EtOH : ethanol

MIC : minimum inhibitory concentration

DPPH : 2,2-diphenyl-1-picrylhydrazyl

4. Essential oil from *Citrus* spp

Natural flavors and fragrances are the odoriferous principles found in various parts of the plant including the seeds, roots, wood, bark, leaves, flower, fruits, balsam and resin. They are called essential oils because they represent the characteristic essence of their origin. The responsible chemicals for the flavor or aroma are organoleptic compounds, which affect the sense organs. They present in their sources at various concentration levels ranging from part per billion to part per hundred. These compounds have molecular weights normally below 300 amu and are relatively volatile.

Essential oils or aroma chemicals have greatly different in their chemical constitutions, but they have some common characteristic physical properties, such as refractive index, optical activity, immiscibility with water, and sufficient solubility to impart aroma to water. They are soluble in ether, alcohol, organic solvents, as well as supercritical carbon dioxide.

Essential oils are volatile oils that differ from nonvolatile fixed oils, i.e., glycerides of fatty acids. These essential oils can be classified into :

1. Acyclic monoterpene hydrocarbons such as myrcene and ocimene.
2. Cyclic monoterpene hydrocarbons such as p-cymene, pinene and sabinene.
3. Acyclic oxygenated monoterpenes such as farnesol, linalool and neral.

4. Cyclic oxygenated monoterpenes such as terpineol and geraniol.
5. Acyclic sesquiterpene hydrocarbon such as farnesene
6. Cyclic sesquiterpene hydrocarbon such as copaene and humulene.
7. Acyclic oxygenated sesquiterpenes such as nerolidol.
8. Cyclic oxygenated sesquiterpenes such as nootkatone and spathulenol.
9. Aromatic compounds such as indole.
10. Long chain hydrocarbons such as tetradecanal and dodecanal.

Terpene hydrocarbons (monoterpenes and sesquiterpenes) are the hydrocarbons derive from isoprene unit (2-methyl butadiene) having molecular formular of $(C_5H_8)_n$ (monoterpene hydrocarbons contain n = 2 and sesquiterpene hydrocarbon contain n=3). They are the unsaturated compounds that can decompose by hydrolysis or photolysis and change to the other compounds.

The odor and taste of essential oils are mainly determined by these oxygenated constituents, which some extent is soluble in water, and most of them are soluble in alcohol.[4, 82]

The essential oils from *Citrus* spp. (fruit peel, leaf and flower) are well known as the perfumery and flavoring agent from natural source and have been produced in metric tons per year. The quality of Citrus essential oils obviously depends on a large extent of factors deriving from the nature of itself (provenance, type of soil, climate, Citrus variety), but the processing of raw material also has a significant effect. Since the fragrance of an essential oil is directly related to the content of aldehydes and esters, then extraction technique is very important. In order to produce Citrus oil with a high aldehyde content, the amount of water used in the procedures should be reduced to the minimum as necessary. The chemical transformations of Citrus oil which may occur during the distillation method, by subject to high temperatures (95-105 $^{\circ}$C) for long periods of time (6-12 hours) in a very acidic environment (pH 2.2-2.4) can modify the Citrus oil in its composition : aldehyde content (neral, geranial) and sabinene almost disappear, otherwise, 1,4- and 1,8-cineole, terpinolene, α-terpineol and *p*-cymene are formed.[4]

4.1 Preparation of Citrus essential oils [4, 82]

4.1.1. Cold pressing method

Cold pressing is the method specifically used for recovering essential oils from peel of *Citrus* spp. There are three distinct types of phenomena occurring during the process.

a. The oil glands that contain essential oil, located in the epidermis layer, are ruptured.

b. The creation in the peel of compressed areas surrounded by areas which are under less pressure, through which the oil can be expressed.

c. The abrasion of the peel.

The abrasion of the peel allows the oil to ooze out from the outside surface in the form of liquid that is separated rapidly after standing into three layers. The upper layer is practically pure essential oil; the intermediate layer contains emulsified oil and includes colloidal particles in suspension; and the lower aqueous layer contains no oil and cannot be used. Recovering of the essential oil could be done by centrifugation. The essential oil that gains from this method may contain traces of water, which will induce slow hydrolysis. The water should be eliminated by treating with anhydrous sodium sulphate.

Cold pressing Citrus oils have superior odor characteristics when compared to steam distilled oils, because they derive from the non-thermal processing. These oils are stable because of the presence of natural antioxidants such as tocopherol, which is not degradation by heat. However, these oils contain plenty of unsaturated components, such as waxy and terpenes, which can polymerise easily to resinous materials, thus producing alteration of their aroma and darkness in color. That is why during storage, the deposited waxy and resin residue are found on the bottom of the essential oil container.

4.1.2. Steam distillation

Distillation is the process of distilling plant material with steam generated outside of the still in a steam generator that generally referred to as a boiler. As in steam and water distillation, the plant material is supported on a perforated grid above the steam inlet. Recovery of the essential oil is facilitated by distillation of two immiscible liquids, namely water and essential oil and based on the boiling temperature, the combined vapor pressures equaling to the ambient pressure. Thus the essential oil ingredients whose boiling points are normally range from 200 to 300 $^{\circ}$C are made to boil at a temperature closing to that of water. In case of the lighter than water oils, they floats at the top and water goes to the bottom. Essential oils which are difficult to volatile in steam are mostly left behind in the still with the botanical matter, while some very volatile chemicals may be lost in the distillation process. The steam distillation process is the most widely acceptable process for large scale of essential oils production. However, an obvious drawback of this process may be the induction of chemicals changes by oxidation and hydrolysation reaction, so that, the recovered oils are often difference from those presence in the original source. Furthermore, if distillation is carried out under reduced pressure, vacuum steam distillation, the consequent lowering of the boiling point significantly helps to avoid, or reduce, harmful reactions to the integrity of the original composition of the essential oil and allows recovery of components which are not distilled under ordinary pressure condition.

Distilled essential oil should be almost colorless. The yellow color indicates that the distillation has been carried out too quickly or too slowly. If distillation is interrupted during processing, the quality of recovered essential oil is poor with high amount of hydrocarbons and deficiency in oxygenated compounds.

4.1.3. Enfleurage

Enfleurage is the classical method of flower and aromatic botanicals extraction for perfumery and aromatherapy using cold fats or lard as extractant. This process is suitable for flower which continue to emit fragrance even after plucking. The odorless fat is spread on glass plates which are placed in closed system. The flower are put in the system and replaced by the fresh one everyday until the fat is

saturated with essential oil. The saturated fat is dissolved in alcohol at 30 to 40 °C and then cooled to 5 to 10 °C which the fat is precipitated out. The filtrate which is concentrated under vacuum to eliminate alcohol and the liquid residue is called absolute. This process consumes extensive time, labor and other steps to eliminate alcohol and fat. However, the essential oil receiving from this method is superior aroma since it is non-thermo labile process.

4.1.4. Solvent extraction

These processes use hydrocarbon solvents, organic solvents, such as hexane, petroleum ether, benzene, toluene, ethanol, isopropanol, ethyl acetate, acetone, etc., to extract plant materials. The extraction occurring by diffusion transfers from the solids to the surrounding solvents, known as leaching. The operating temperature and time of extraction are specific to the nature of the botanical substances and contact devices. The concentrated solution is done by vacuum distillation. The residue, dark colored and waxy substance, is called concrete. The concrete is dissolved in alcohol at 30 to 40 °C and then cooled to 5 to 10 °C which the wax precipitates out. After filtration, the filtrate is concentrated under vacuum to eliminate alcohol and the liquid residue which is called absolute.

As described above, there are many steps involved in recovering good quality and quantity of fragrance from botanical substances. The fragrance may lose of top notes, vary of volatile compounds, and contain some undesirable impurities, depending on the polarity of the solvents. The thermal degradation, hydrolysis, alcoholysis, etc. may happen and could affect the quality and stability of oils. The important process of this method is the solvents elimination from extracts, because of their harmfulness.

4.1.5. Supercritical carbon dioxide extraction (SCO_2)[82]

When a gas is compressed to a sufficiently high pressure, it becomes liquid. If the gas is heated to a specific temperature, at the specific pressure, the hot gas will become supercritical fluid. This temperature is called the critical temperature and the corresponding vapor pressure is called the critical pressure. The values of the

temperature and pressure are defined as critical point which is unique to a given substance. These state of the substances are called supercritical fluid when both the temperature and pressure exceed the critical point values as shown in a pressure-temperature phase diagram (Figure 2). This fluid now takes on several of gas and liquid properties. Supercritical fluid is the region where the maximum solvent capacity and the largest variations in solvent properties can be achieved with small changes in temperature and pressure. It offers very attractive extraction characteristics, owing to its favorable diffusivity, viscosity, surface tension and other physical properties. Its diffusivity is one to two orders of magnitude higher than those of liquids. The diffuseness facilitates rapid mass transfer and faster completion of extraction than conventional liquid solvents. The low viscosity and surface tension enable it to easily penetrate the botanical materials from which the active components are extracted. The gas-like characteristics of supercritical fluid provide ideal conditions for extraction of solutes giving a high degree of recovery in a short period of time.

The most desirable supercritical fluid solvent for extraction of natural products is carbon dioxide (CO_2). It is an inert, inexpensive, easily available, odorless, tasteless, environment-friendly, and generally regards as safe solvent. In the supercritical fluid processing with CO_2, there is no solvent residue in the extract, because of it becomes gas in the ambient condition. Its near-ambient critical temperature, 31.1 $^{\circ}$C, makes it ideally suitable for thermolabile natural products extraction. Due to its low latent heat of vaporization, low energy input is required for the extraction separation system. SCO_2 produces the most natural smelling extracts, since the hydrolysis does not occur in the process.[4]

The advantages of the SCO_2 technique are well known by now and it is often regards as an alternative to the classical methods. It has been established as an environmental friendly technique for separating essential oil from herbs or plants. The chemical compositions of *C. maxima* peel oil from classical techniques, cold pressing and distillation, have been the subject of many studies but they have no report about chemical constituents of *C. maxima* by SCO_2 before. There was only one study about SCO_2 condition (pressure, temperature and period) of *C. maxima* peel in the

laboratory scale. This extraction condition was 40°C, 13 MPa and duration of extraction was 1 hour.[83]

CHAPTER III
MATERIALS AND METHODS

1. Plant materials

The fruit of *Citrus maxima* cultivar khao–yai were collected from Bangkontee District, Samutsongkarm Province, Thailand in December 2005 for their peel, while their flower were collected in February 2006 (Figure 3). Neroli essential oil, essential oil from Italy, which has been used as standard, was extracted by solvents extraction method and was purchased from Topica Life company, Thailand.

C. maxima peel (flavedo) were divided into two parts. The first part, was prepared for supercritical carbon dioxide extraction (SCO_2). It was dried with hot air oven (35 to 40°C, Figure 4) and milled with a cutting mill (Taian Hkeb-11, D.O.L. Magnetic starter) to minimize the approximate particle size 0.5 cm. The second fresh part (Figure 5) was distilled by vacuum steam distillation (VSD) and pressed after peeling by cold pressing method (CPM). The fresh flower, which were extracted by SCO_2, were processed as soon as they were collected.

2. Extraction methods

2.1 Supercritical carbon dioxide extraction (SCO_2)

This research used the pilot scale SCO_2 (Guangzhou Masson New Separation Technology, China) which is belong to Thai Traditional and Herbal Development Center (TTHD), where located at module 1, Biotechnology Pilot Plant, Thailand Science Park, Klong 1, Klongluang, Pathumtani, Thailand (Figure 6). The plant material was loaded in to a high pressure stainless steel extractor tank and the extract-laden carbon dioxide was sent to the two separator tanks. Samples eight kg. of prepared peel as described above, were extracted with supercritical carbon dioxide and the experiments for optimization the extraction conditions were carried out. The evaluated parameters were temperature and pressure of the extractor (40 to 60 °C and 13 to 20 MPa) and of two separators (20 to 40 °C and 5 to 8 MPa). Another variables

were kept constant as particle size of plant material, carbon dioxide flow rate (1.0 ml/min), time for equilibrium condition (30 minutes) and extraction time (3.0 hours). The best established temperature and pressure conditions were selected and used in the experiments for separation the essential oil from plant materials. For finding the best condition for extraction essential oil from *C. maxima* flower, the cut fresh peel, the milled dried pills, the milled fresh flower and whole fresh flower were used for trial and error. Fresh flower dipping in the SCO_2 for 24 hours, prior extraction, were used for comparing the result.

From the experiments, the best extraction condition gave the oleoresin deposit in the first separator and the second separator contained two immiscible phase of essential oils and water. The essential oils were separated, filtered and then anhydrous sodium sulphate was added for elimination of trace water. In this study the peel essential oil that derived from SCO_2 was called SCP and the flower essential oil that derived from SCO_2 was called SC-f.

2.2 Vacuum steam distillation (VSD)

The fresh *C. maxima* peel (10.0 kgs) were cut in the small size (about 1.0 to 3.0 cm.) and extracted by VSD apparatus, Figure 7 (Pilot scale, Model : T-36, Technical Business Company, Thailand located in TTHD). The peel were submitted to steam distillation for 3.0 hours, under controlled temperature and pressure (about -600 bar and 60 $^{\circ}$C). The extracted essential oil, called VP, was dried over anhydrous sodium sulphlate before storage.

2.3 Cold pressing method (CPM)

The fresh *C. maxima* peel (1.0 kg) were cut in the small size (1.0 to 3.0 cm). The cut peel were fed into the screw pressing instrument, vegetable juicer, which was household used for producing vegetable and fruit juice. During pressing, the liquid was separated and flowed out through the net, while the pressed peel (pulp) was ejected from the front end of the instrument. The mixture of oil and water was separated and recovered by centrifugation with 3000 rpm for 10.0 mins. Then, the oil was separated, filterted and anhydrous sodium sulphate was added. For this study the oil derived form cold pressing method was called CP.

All of the oil samples, SCP, SC-f, VP, CP, neroli, were kept in air tight, protected from light containers and placed in refrigerated at +4 °C prior to analysis.

3. Analysis

Samples (Figure 8 to 12)

SCP : Essential oil derived from *Citrus maxima* peel, extracted by SCO_2

CP : Essential oil derived from *Citrus maxima* peel, extracted by CPM

VP : Essential oil derived from *Citrus maxima* peel, extracted by VSD

SC-f : Essential oil derived from *Citrus maxima* flower, extracted by SCO_2

Neroli : Essential oil derived from *Citrus aurantium* var. *amara* flower, extracted by organic solvent, from Italy (Commercial product)

3.1 Physical properties

3.1.1 Density

The true density of essential oils were measured using the pycnometer and analytical balance (Sartorius BP 210S) at the Department of Pharmaceutical chemistry, Faculty of Pharmacy, Silpakorn University.

3.1.2 Optical rotation $[\alpha]_D$

The angle of rotation of all pure essential oils were measured on Polarimeter (Jasco P-1010, Japan) at the Department of Chemistry, Faculty of Sciences, Silpakorn Univesity. The wavelength of the sodium D line (589 nm) and temperature at 30°C were conditions that used for experiment.

3.1.3 Refractive Index $[n]_D$

The refractive index of all pure essential oils at 25°C were measured by Refractometry (Schmidt & Haensch berlin, Germany) at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Silpakorn University.

3.2 Chemical Characteristic

3.2.1 Infrared absorption spectra (IR spectrum)

IR spectrums (fingerprints) of all essential oils were determined by Fourier transform - Infrared Spectroscopy (Nicolet 4700 FT-IR Spectrometer,) at Faculty of Pharmacy, Silpakorn University. The IR spectra were reported in % reflectance.

3.2.2 Gas Chromatography (GC)

Chemical identification and quantificaation of essential oils were investigated by Gas Chromatography equipped with FID detector (Agilent Technologies 5890 series II, USA) at Faculty of Pharmacy, Silpakorn University. Two types of capillary columns, DB-5 and cabowax, were used for separation of different profile volatile components. The operating condition of GC was shown in Table 3 and the carrier gas was Helium 99.9999% purity, Flame Ionization Detector (FID), DB-5 : J&W, Agilent Technologies, USA, Carbowax : Ohiovalley specialty, USA.

The identification of individual components was based on the comparison of their retention index (RI), Kovats index (KI), on polar and non-polar column, and their mass spectra from GC-MS.

In this study, the GC methods that were employed for analysis were validated to confirm the analytical results. The validate test methods that were done were selectivity, linearity, accuracy and precision.[84]

Selectivity

Selectivity was a measure of the capability of the analytical method to determine a particular analyte in various matrices with minimal or no interference from other matrix components. For choosing the operating condition of GC, VP was used to be the test sample for analysis, because it has been known as pure volatile oil. Pure VP, VP mixed with solvent and VP mixed with all standard solutions and

internal standard were injected into GC. Three evaluated parameters were tested : ramp (2.0, 3.0, 4.0, 5.0°C/min), column flow (1.0, 2.0 ml/min) and split ratio (1 : 20, 1 : 90, 1 : 120). Another variables were kept constant, for example, initial temperature, final temperature and injection temperature. The best conditions were defined by the ability of condition to resolve individual compounds that could be measured using a single procedure.

Calibration linearity

Calibration of the gas chromatography delineated the relationship between the detector response and the concentrations of the analyte introduced into instrument. A calibration curve was the graphical depiction of this relationship. Calibration curve of a gas chromatographic method involves the analysis of standards containing target compounds at different concentrations covering the working range of the instrument. In order to produce calibration curve for this work, standard solutions : *R*-(+)-limonene, linalool, nerolidol and farnesol were the target compounds. The standard solutions were prepared as analyte solutions at 7 concentration levels, 7 repeated of each concentration for limonene and linalool (0.0001, 0.001, 0.01, 0.1, 0.3, 0.5, 0.9 µl/µl) and 5 concentration levels, 7 repeated for nerolidol, farnesol (0.0001, 0.001, 0.01, 0.05, 0.1 µl/µl), based on the amount of target compounds in the sample. Acceptability of linearity data was often judged by examining the correlation coefficient of the linear regression line for the response versus concentration. A correlation coefficient (r^2) of more than 0.999 was generally considered acceptable. The quantitation of target compounds could be calculated using the equation from linear correlation.

Accuracy

Accuracy was how close the experimental data is to the true value. Standards addition techniques and matrix spikes would be used and recoveries determined. The standard addition techniques indicated how the method performed with respect to accuracy of the final procedural steps. The accuracy should be evaluated at the low as well as the high analyte concentration values expected. An accuracy criterion for a particular method is that the mean recovery value would be 95 $\pm$ 2% at each

concentration over 80 – 120% of the anlyte range concentration. This study used the standard addition techniques by adding linalool into VP in 4 concentrations (0.002, 0.004, 0.006, 0.008 µl/ml) and the accuracy was determined on DB-5 column. *R*-(+)-limonene was added into neroli oil in the same concentrations of linalool and the accuracy was determined on carbowax column. Each experiment was analysed in triplicate.

Precision

Precision of GC method was the measurement of closeness of analyte concentrations to each other when the analytes were performed on identical conditions, for example, the same method, same sample, same operator, and same laboratory conditions over a short period of time, called repeatability. The precision data was calculated and expressed as standard deviation (SD) which often reported as the relative standard deviation (RSD). The precision data were obtained from the multiple injections of standard addition sample solutions. Each sample was injected in to GC in triplicate. The precision criteria for GC would be that the instrument precision (RSD) should be $\pm$ 1%, and the repeatability of the method would be $\pm$ 2%.

For Retention Index (RI) or Kovats index (KI) calculation, each essential oil samples (pure) were injected into GC for 7 repetition and the retention times were averaged and used for calculation of KI. All essential oil samples were also investigated by GC-MS for mass spectrum.

Table 3 The operating condition of GC

Operating condition	DB-5	Carbowax
Capillary column		
Compositions	5%phenyl & 95% dimethylpolysiloxane	polyethylene glycol
Length	30 m.	60 m.
Thickness	0.25 µm	0.25 µm
Diameter	0.25 µm	0.25 µm
Carrier gas	Helium	Helium
Column flow rate	1.0 ml/min	2.0 ml/min
Carrier gas pressure	12.5 Psi	33.5 Psi
Oven		
Initial temperature	60.0°C (5.0 mins)	60.0°C (5.0 mins)
Ramp	3.0°C/min	3.0°C/min
Final temperature	250.0°C (15.0 mins)	180.0°C (30.0 mins)
Injection		
Injection temperature	230°C	230°C
Split flow	90.0 ml/min	90.0 ml/min
Split ratio	1 : 90	1 : 90
Sample volume	1.0 µl	1.0 µl
Detector		
Type	FID	FID
Detector interface temperature	240°C	240°C

3.2.3 Gas Chromatography-Mass Spectroscopy (GC-MS)

Mass spectra of each chemical constituents in essential oil samples were used to confirmed the identification. They were compared with quadrupole mass spectra in the library of HP-Chemstation data processor, library database for mass spectra matching up to 80% : Willey 7n, NIST02 and Pesticide and compared with the mass spectra as reported by R.P. Adams, 2001.[85] GC-MS was performed with a Agilent Technologies GC 6890, USA gas chromatograph, equipped with a Agilent Technologies MSD 5973N mass spectrometer as detector, USA. The instrument is

located at Faculty of Pharmacy, Silpakorn University. The operation of GC-MS was shown in Table 4.

Table 4 The operating condition of GC-MS

Operating condition	DB-5	Carbowax
Capillary column		
Compositions	5%phenyl & 95% dimethylpolysiloxane	polyethylene glycol
Length	30 m.	60 m.
Thickness	0.25 µm	0.25 µm
Diameter	0.25 µm	0.25 µm
Carrier gas	Helium	Helium
Column flow rate	1.0 ml/min	2.0 ml/min
Carrier gas pressure	12.5 Psi	33.5 Psi
Oven		
Initial temperature	60.0°C (5.0 mins)	60.0°C (5.0 mins)
Ramp	3.0°C/min	3.0°C/min
Final temperature	250.0°C (15.0 mins)	180.0°C (30.0 mins)
Injection		
Injection temperature	230°C	230°C
Split flow	90.0 ml/min	90.0 ml/min
Split ratio	1 : 90	1 : 90
Sample volume	1.0 µl	1.0 µl
Detector		
Type	Ms	Ms
Detector interface temperature	240°C	240°C
Solvent delayed	3.0 mins	3.0 mins
Electron energy	70.0 eV (EI)	70.0 eV (EI)
Mass spectra matching	up to 80%	up to 80%

Standard compounds for GC and GC-MS

(±)-Linalool	:	95.0%, Fluka®, Switzerland. density 0.861, 100.0 gm
(*R*)-(+)-Limonene	:	98.0%, Aldrich®, Germany. density 0.840, 100.0 gm
Nerolidol	:	98.0%, Aldrich®, Germany. density 0.875, 25.0 gm
Farnesol	:	95.0%, Aldrich®, USA. density 0.886, 25.0 gm (mixture of isomers)
Alkane C_8 to C_{20}	:	40.0 µg/ml in hexane, Fluka®, Germany, 5.0 ml
Alkane C_{21} to C_{40}	:	40.0 µg/ml in toluene, Fluka®, Germany.5.0 ml

Solvent	:	99.0% *n*-Hexane, AnalaR®, BDHL Laboratory Supplies,2.5 L
Internal standard	:	Tetradecane olefine free 99.5%, Fluka®, Germany, 5.0 ml

4. Anti-microbial activity

The anti-microbial activities of *C. maxima* peel and flower essential oil from different extraction methods (SCP, CP, VP and SC-f) were investigated in this study using the broth microdilution technique. Three test organisms were used in the assay.

4.1 Assay medium requirements

Tryptic soy broth (TSB) media (USP 211825) and Muller Hinton broth (MHB) media (USP 275730) were used as nutrient sources for the test organisms at the concentration of 3.0 gm/ml and 2.1 gm/ml, respectively. All media and equipments were sterilized by autoclave before using.

4.2 Test microorganisms

Three test organisms, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739 and *Candida albicans* ATCC 17110, were used for the assay. The microorganisms were maintained in Tryptic soy agar (TSA) for bacterias and Sabouraud dextrose agar (SDA) for yeast at 37.0°C for 18-24 hours. The isolated colony was incubated to broth and incubated overnight at 37.0°C. One loop of this cultured broth was transferred to new tube and resuspended in TSB, then incubated at 37.0°C for 2-5 hours in the shaking incubator. After 2-5 hours incubation, the microorganism culture was used for the assay.

4.3 Microdilution plate

The 96 well cell culture cluster, round bottom with lid, sterile (COSTAR®; Corning Incorporated 3799, USA) was used.

4.4 Microplate reader

Packard/A Packard BioScience Company, Fusion™, USA.

Plate type	96 well plate
Detection mode	Absorbance
Well read time (seconds)	1.0
Number of times to read each well	1.0
Light source type	Halogen CW
Light source intensity	10.0

Primary excitation filter position	Abs 550
Primary emission filter position	Diffuser

4.5 Mircodilution method for anti-microbial assay

The amounts of *S. aureus, E. coli* and *C. albican*s were adjusted by comparing the turbidity to the McFarland suspension No.5 (0.5% BaCl2 in 0.56 N in SO_4 v/v) and further diluted to be 10^6 CFU/ml using MHB as diluent. To overcome the insolubility of the oils in the broth, the assay was performed in 1% DMSO of MHB. The 90.0 µl of five folded dilutions of each oil sample were prepared in a 96-well plate. The 10.0 µl of each test organism was added into each well to make a final concentration of approximately 10^5 CFU/ml. In each test, test organism in MHB was used as the positive growth control, while the oil samples in MHB and MHB alone were used as negative growth control. The plates were then incubated at 37 °C for 18 hours. The minimum inhibitory concentration (MIC) value was defined as the lowest concentration of essential oil inhibiting visible growth by using microplate reader. To evaluate whether this MIC of oil could kill the microorganism, 10 µl of each well at MIC was transferred to a new well containing 90 µl MHB and incubated at 37 °C for 18-48 hours. If the oil can kill microbial, the clear solution was found in each well. Each experiment was performed in triplicate.

CHAPTER IV
RESULTS AND DISCUSSION

1. Extraction

In order to obtain the best conditions for the SCO_2 of *C. maxima* peel and flower, the experiments were performed at different pressure-temperature settings of extractor and separators. The best condition for peel and flower extraction were 40°C, 13 MPa for extractor, 40°C, 8 MPa for the first separator, and 20°C, 5 MPa for the second separator. The SCP and SC-f were clear solution with dark-green and light-yellow in color, respectively. The SCP and SC-f were condensed out in second separator with water, while the other compounds of peel and flower were separated in first separator. For SCO_2 of peel, only dry milled peel extraction gave essential oil, while the milled fresh peel could not. Because high water content in fresh peel reduced extraction power of SCO_2.

The extraction of cut fresh peel by SCO_2 in addition of 1% cosolvent (95% ethanol) was given the liquid mixture of ethanol, water, essential oil and both hydrophobic and hydrophilic impurities. The water and ethanol could be eliminate from the mixture by rotary evaporator. However, this evaporation caused the losing of some volatile oil and could not separate the oil from nonvolatile impurities. So the air dried peel (flavodo) were milled to around 0.5 cm in diameter and immediately extraction by SCO_2 under the mentioned condition. The obtained SCP was similar in color and smell to the CP.

The CP was clear solution with dark-green in color. It showed small amount of precipitate at the bottom of the container after storage. The oil that obtained from VSD method was clear solution with no color. Its percentage yield was about 0.2% v/w on fresh peel basis.

In this study the cold press extraction was done by modification of small vegetable juicer which the juice was extracted and the pulp was automatic ejection. The principle of this juice maker was the expression the juice from pulp. The

collected juice was centrifuged for oil separation. The estimated %yield of CP was 0.3% v/w on fresh peel basis.

The present investigation, peel oils from SCO_2 and VSD were gained from pilot scale equipment that required at least around 8-10 kg of the peel per each time of extraction. Because of large amount of raw material require, the criteria for extraction condition selection was the separation of oil from impurities, not the highest yield of oil.

The limitation of *C. maxima* flower oil extraction by SCO_2 was the supply of raw materials (flower). In this study, around 1 kg of fresh *C. maxima* flower were extracted under the best condition that was chosen from *C. maxima* peel oil extraction. The whole flower were extracted in fresh condition, since the smell of dried flower was distorted from their original and their aroma was decreased by the time and method of drying. The only factor of extraction condition of *C. maxima* flower that differ from those of peel was the extraction time. The whole fresh flower were allowed to contact CO_2 for 24 hours in extractor. In commercial, the fresh bitter orange flower that were dipped in water 24 hours for storage before distillation gave the raise of yield about 0.2-0.3% over the fresh flower without dipping in water prior to distillation.

The milled fresh *C. maxima* flower were also on trial for increasing yield of extraction. However, the flower oil could not collect and lots of impurities contaminated in both separators. Then the reduction of flower size could not enhance the yield of the oil. The intensification of particle area by reducing particle size could magnify the mass transfer of raw material to CO_2. This was the reason why lots of impurities were found in the extract. Normally, water was not only reducing mass transfer by decreasing the lipophilicity of CO_2 and impeding the penetration of CO_2 into raw material particles, but also enhancing the solubility of some substance as cosolvent and increasing the hydrophilicity of CO_2. For the extraction of milled fresh flower, water may enhance the power of CO_2 extraction, then lots of impurities were coextracted.

The percentage yield of SCP and SC-f were about 0.4% v/w on dry peel basis and 0.2% v/w on fresh flower basis, respectively. The amount of essential oils of *C. maxima* peel and flower from this study were not the maximum amount. Because

the aims of this study were to find out the extraction conditions that could separated the oils from the impurities and to compare the chemical constituents of the oils from different extraction methods. To the best of our knowledge, this is the first report that the *C. maxima* flower were extracted by SCO_2. There were some previous report about the amount of *C. maxima* peel oil from CPM and steam distillation which were 1.16% and 2.39% on the fresh peel basis, respectively. From this study the *C. maxima* flower oil from SCO_2 extraction method gave 20 times higher amount than that gained from steam distillation of *C. maxima* flower that was reported by Vietnam researcher.

2. Physical and chemical characteristics

Physical values and Infrared spectra (IR spectra) of SCP, CP, VP, SC-f and neroli were shown in Table 5 and Figure 19-23. The density and refractive index of *C. maxima* peel and flower oils and neroli were close to each other. Their density was around 0.8433-0.9445 g/ml and their refractive index was about 1.4622-1.4685. Their optical rotation were vary from +6.975 to +9.369. From the values of density, optical rotation and refractive index and the IR spectrums of all kind of oils that were very close to each other meant that the chemical components in the oils might be nearly the same, but different in proportion.

Table 5 Physical properties of essential oils from *C. maxima* peel and flower, and neroli

Properties	SCP	CP	VP	SC-f	neroli
Density (gm/ml)	0.8803	0.8881	0.8433	0.8500	0.9445
Optical rotation $[\alpha]_D$	+6.975	+7.821	+9.369	+6.975	+0.875
Refractive index $[n]_D$	1.4667	1.4622	1.4685	1.4632	1.4647
Appearance	clear solution	clear solution	clear solution	clear solution	clear solution
Color	dark-green	dark-green	no color	yellow	yellow

3. GC method validation and quantification

3.1 GC method validation

The GC methods of essential oil analysis of both DB-5 and carbowax columns were validated as shown in Table 6. The calibration curves of both columns (Figure 13 to 18) showed the linearity with r^2 = 0.9992 and 0.9922 for DB-5 and carbowax, respectively. The validation data showed acceptable results for accuracy of both capillary columns (DB-5 : 99.10%, carbowax : 102.20%) while the repeatability shown %RSD higher than standard criteria[104] (5.39, 2.80% for DB-5 and carbowax, respectively). For solving this problem, each pure essential oil samples were injected into GC for 7 repetition. For identification, the retention times were averaged before calculated KI and confirmed with mass spectrum by GC-MS.

The chemical constituents of all essential oil samples were identified based on their mass spectra compared with the standard spectra from R.P. Adam[85] and relative index (RI) or Kovats index (KI) from previous reports.[85,87,88,89,90] Kovats index[85, 91] were computed by using the following formula.

$$KI = 100 \{ [(\log R_t(x) - \log P_z) / (\log R_t(P_{z+1}) - \log R_t(P_z)] + z \},$$

where : The $R_t(x)$ is the adjusted retention time of the analyte. It was measured at the same conditions as those of the *n*-alkanes (C_8-C_{40}) and then compared with the two *n*-alkanes which eluted in front and behind (containing “z” and “z+1” carbon atoms respectively) and P is the adjusted retention time of *n*-alkane which calculated by the same as the adjusted retention time of analytes:

$$R_t(x) = R_t(x_{average}) - R_t(\text{air peak}),$$

where : $R_t(x_{average})$ is the average retention time of analyte , R_t(air peak) is the average retention time of air peak.

The quantity of each identified chemical constituent was determined by area normalization method as followed

$$\% \text{ area of compound A} = \frac{\text{area under peak A} \times 100}{\text{total area of all peaks}}$$

The qualitative and quantitative analysis of *C. maxima* peel and flower oil using DB-5 were shown in Table 8 and 12. The qualitative and quantitative analysis of *C. maxima* peel and flower oil using carbowax were shown in Table 9 and 13.

3.2 Quantification

The compounds in *C. maxima* oils and neroli that were chosen for quantitative analysis were *R*-(+)-limonene, linalool, nerolidol and farnesol. These compounds were the prominent compounds in essential oil of *C. maxima* flower oil (steam distillation) of Vietnam (18.2, 16.4, 29.3 and 15.7%, respectively).[60] As shown in Table 13 and 14, *C. maxima* flower which were extracted by SCO_2 (SC-f) had the different amount of these four compounds : 86.20, 0.38, 0.59, 0.95%, respectively, using DB-5 capillary column. The result showed that SC-f have higher amount of *R*-(+)-limonene but lower amount of total oxygenated compounds than *C. maxima* flower essential oil from Vietnam. These may happen from different variety, extraction methods and cultivated area of *C. maxima*. To analyse the quantity of these four compounds that contained in the essential oil samples (SCP, CP, VP, SC-f and neroli), the standard curves of them were ploted using DB-5 capillary column and the amount of each compound in ng/µl have been shown in the Table 7. The VP contained the highest amount of *R*-(+)-limonene (833.56 ng/µl), neroli contained the highest amount of linalool (158.62 ng/µl), while the highest amount of (*E*)-nerolidol and (*E*, *E*)-farnesol were found in SC-f (4.20 and 5.35 ng/µl, respectively) compared with SCP and CP. The other isomers of farnesol were found only in SCP and SC-f. The SC-f had a little bit higher amount of farnesol isomer than SCP. It was interesting that CP, VP and neroli had not faenesol and VP did not contain nerolidol. These

implied that when the peel and flower were extracted by using CPM, VSD and solvent extraction methods, the small amount chemicals could be lost.

Table 6 Result of method validation for GC

Column	Standard	r^2	% Recovery	% RSD
DB-5	Linalool	0.9992	99.10	5.39
Carbowax	*R*-(+)-Limonene	0.9922	102.20	2.80

Table 7 The amount of some important compounds in essential oil samples analysed by using DB-5 capillary column and GC

Compounds	Amount (ng/µl)				
	SCP	CP	VP	SC-f	neroli
R-(+)-limonene	621.31	699.58	833.56	446.63	8.82
Linalool	4.17	3.75	3.92	5.25	158.62
(*E*)-nerolidol	0.57	0.40	-	4.20	0.48
(*Z*, *E*)-farnesol	0.33	-	-	0.57	-
(*Z*, *Z*)-farnesol	0.29	-	-	0.68	-
(*E*, *E*)-farnesol	0.36	0.32	-	5.35	-
(*E*, *Z*)-farnesol	0.29	-	-	0.62	-

Table 8 Qualitative and quantitative analysis of *C. maxima* peel essential oil analysed by using DB-5 capillary column

Peak no.[a]	KI		Components[c]	% Area		
	Reference[b]	Sample		SCP	CP	VP
1	930	934	α -thujene	trace	trace	trace
2	939	940	α -(-)-pinene	0.40	0.51	0.39
3	955	957	camphene	trace	trace	trace
4	975	978	sabinene	0.35	0.32	0.24
5	979	981	β-(+)-pinene	0.54	1.08	0.37
6	991	993	myrcene	1.71	1.93	1.80
7	1003	1006	α -phellandrene	0.42	trace	0.55
8	1029	1025	*R*-(+)-limonene	93.74	95.33	95.41
9	1050	1054	(*E*)-β-ocimene	0.06	0.01	0.08
10	1060	1064	γ -terpinene	trace	trace	trace
11	1070	1073	(*Z*)-sabinene hydrate	trace	trace	trace
12	1089	1091	terpinolene	trace	trace	0.01
13	1097	1095	linalool	0.17	0.10	0.10
14	1137	1136	(*Z*)-limonene oxide	0.02	trace	0.01
15	1142	1141	(*E*)-limonene oxide	0.02	trace	trace
16	1153	1155	citronellal	trace	trace	trace
17	1189	1191	α -terpineol	0.16	0.06	0.06
18	1199	1202	γ -terpineol	0.05	trace	0.04
19	1202	1206	*n*-decanal	0.03	trace	0.02
20	1217	1219	(*E*)-(+)-carveol	0.03	trace	0.01
21	1230	1229	nerol	0.03	0.01	0.03
22	1229	1232	*Z* -(+)-carveol	0.03	0.10	0.01
23	1238	1242	neral	0.14	0.02	0.09
24	1253	1257	geraniol	0.03	0.14	0.02
25	1267	1271	geranial	0.23	0.01	0.12
26	1272	1276	perilla aldehyde	0.03	trace	trace
27	1291	1292	indole	0.04	trace	trace
28	1337	1334	methyl antranilate	0.01	trace	trace
29	1349	1342	α -terpinyl acetate	0.04	0.01	trace
30	1362	1365	neryl acetate	0.03	0.01	0.01
31	1377	1381	α -copaene	0.01	0.01	trace
32	1381	1383	geranyl acetate	0.12	0.06	0.03
33	1388	1397	β-(-)-cubebene	0.02	no	no
34	1409	1410	dodecanal	0.06	no	0.02
35	Ms	1412	unknown from lime oil	0.02	0.04	trace
36	1419	1425	β-caryophyllene	0.16	0.04	0.08
37	1432	1437	β-copaene	0.01	0.02	trace
38	1455	1460	α -humulene	0.02	trace	trace
39	1485	1487	germacrene D	1.04	0.02	0.38
40	1498	1501	α -(-)-selinene	0.11	0.06	0.04
41	1500	1503	bicyclogermacrene	0.03	trace	trace
42	1506	1512	(*E*, *E*)-α -farnesene	0.01	trace	trace
43	1523	1522	δ-(+)-cadinene	trace	trace	trace
44	1561	1564	germacrene B	0.03	trace	trace

Table 8 Qualitative and quantitative analysis of *C. maxima* peel essential oil analysed by using DB-5 capillary column (continued)

Peak no.[a]	KI		Components[c]	% Area		
	Reference[b]	Sample		SCP	CP	VP
45	1563	1566	(*E*)-nerolidol	0.04	0.02	trace
46	1576	1583	germacrene D-4-ol	0.02	no	trace
47	1613	1614	tetradecanal	0.03	no	trace
48	1701	1692	(*Z*, *E*)-farnesol	0.01	no	no
49	1718	1717	(*Z*, *Z*)-farnesol	trace	no	no
50	1725	1726	(*E*, *E*)-farnesol	0.01	0.01	no
51	1746	1748	(*E*, *Z*)-farnesol	trace	no	no
52	1807	1812	(+)-nootkatone	0.06	no	no

[a]Peak numbers refer to Figure 24 - 26

[b]KI from R. Adams[85]

[c]Peak identifications are based on MS comparisions with file spectra and relative retention time(KI)

trace : less than 0.01%

SCP : essential oil of *C. maxima* peel by supercritical carbon dioxide extraction

CP : essential oil of *C. maxima* peel by cold pressing method

VP : essential oil of *C. maxima* peel by vacuum steam distillation

Ms : identification from mass spectra of willey database library and/or R. Adams

no : could not detected

Table 9 Qualitative and quantitative analysis of *C. maxima* peel essential oil analysed by using carbowax capillary column

Peak no.[a]	KI		Components[c]	% Area		
	Reference	Sample		SCP	CP	VP
1	1036[d]	1032	α -(-)-pinene	0.43	0.44	0.42
2	1038[d]	1039	α -thujene	no	0.17	no
3	1078[d]	1074	camphene	trace	0.01	trace
4	1120[d]	1112	β-(+)-pinene	0.49	0.97	0.27
5	1130[e]	1121	sabinene	0.43	0.45	0.36
6	1156[d]	1155	myrcene	2.26	2.01	1.27
7	1173[d]	1163	α -phellandrene	no	no	1.20
8	1210[d]	1219	*R*-(+)-limonene	93.37	95.16	95.24
9	1238[d]	1245	(*E*)-β-ocimene	0.07	0.02	0.09
10	1272[e]	1263	*p*-cymene	0.04	no	0.01
11	1279[d]	1277	terpinolene	0.01	trace	0.01
12	1469[d]	1466	δ-elemene	0.02	0.02	0.01
13	1555[d]	1548	linalool	0.17	0.10	0.11
14	1591[d]	1558	β-(-)-elemene	0.02	0.01	0.03
15	1569[d]	1561	linalyl acetate	0.01	0.01	0.01
16	1562[d]	1588	β-caryophyllene	0.16	0.05	0.09
17	1601[d]	1609	terpinen-4-ol	0.01	trace	0.01
18	1672[d]	1660	α -humulene	0.02	0.01	0.01
19	1680[f]	1683	neral	0.15	0.10	0.09
20	1712[e]	1698	germacrene-D	0.17	0.03	1.42
21	1685[d]	1709	α -terpineol	0.29	0.07	0.08
22	1738[d]	1725	bicyclogermacrene	0.13	0.07	0.04
23	1730[d]	1736	geranial	0.23	0.14	0.12
24	1754[d]	1752	geranyl acetate	0.17	0.07	0.04
25	1756[d]	1753	β-(+)-selinene	0.02	0.01	0.01
26	1765[d]	1775	citronellol	0.02	0.01	trace
27	1818[d]	1788	perilla aldehyde	0.04	0.01	0.01
28	1808[d]	1806	nerol	0.01	0.01	0.02
29	1864[d]	1819	germacrene B	0.03	0.01	0.01
30	1839[d]	1851	(*E*)-(+)-carveol	0.03	0.04	trace
31	1842[d]	1855	geraniol	0.03	0.01	0.02
32	2044[d]	2039	(*E*)-nerolidol	0.01	0.01	trace
33	2153[d]	2144	(+)-spathulenol	0.02	no	no
34	2260[d]	2231	methyl anthanilate	0.02	no	no
35	2250[d]	2254	(+)-nootkatone	0.13	no	no
36	Ms	2274	(Z, *E*)-farnesol	0.01	no	no
37	2371[g]	2411	(*E*, *E*)-farnesol	0.03	no	no

[a]Peak numbers refer to Figure 27 - 29

[c]Peak identifications are based on MS comparisons with file spectra and relative retention time (KI)

[d]KI from N.W. Davies[87]

[e]KI from T. Acree and H. Arn[88]

[f]KI from S.M. Njoroge and other[89]

[g]KI from A.M. El-Sayed[90]

Ms : identified from mass spectrums compare with standard compound from R. Adam [85]

trace : less than 0.01%

SCP : essential oil of *C. maxima* peel by supercritical carbon dioxide extraction

CP : essential oil of *C. maxima* peel by cold pressing method

VP : essential oil of *C. maxima* peel by vacuum steam distillation

no : could not detected

Figure 24 to 29 showed the elution profile of *C. maxima* peel oils from SCP, CP and VP. The identified volatile constituents of these three oils and their % areas are shown in Table 8 and 9, according to their GC elution order. A total number of 52, 44 and 46 constituents were found in SCP, CP and VP, respectively, using DB-5 capillary column analysis. And total number of 35, 30 and 31 constituents were found in SCP, CP and VP, respectively, using carbowax capillary column analysis. The compounds were categorized into acyclic and cyclic monoterpene hydrocarbon, acyclic and cyclic oxygenated oxygenated monoterpenes, acyclic and cyclic sesquiterpene hydrocarbons, acyclic and cyclic oxygenated sesquiterpenes, aromatic compounds, long chain hydrocarbons and miscellaneous (Table 10).

The comparison of % relative area of chemical components in SCP, CP and VP essential oils using two types of capillary column from GC and GC-MS analysis (Table 11) did not show much difference. The first and second of quantity of chemical groups in these oils from high to low by DB-5 and carbowax were cyclic monoterpenes and acyclic monoterpenes, respectively. These three types of oils contained 94.77–97.52% relative amount of cyclic monoterpenes. *R*-(+)-limonene was the most abundance (93.37-95.41%). These amounts closed to the report from Kenya[89] (94.8%) and were higher than those from Japan (67.60%) and Vietnam (86.70%).[60, 92]

The % relative amount of acyclic monoterpenes was 1.35-2.33%. Myrcene was the most abundance (1.27-2.26%). However, the previous reports showed higher amount of myrcene than this study.[60, 89, 92]

The chromatogram of SCP presented more number of peaks than the other methods. It contained some components that could not be detected in other oil samples, for example, (+)-spathulenol, methyl anthanilate, farnesol and (+)-nootkatone. In addition, SCP contained higher amount of total oxygenated compounds than CP and VP. The amount of total oxygenated compounds from DB-5 of SCP, CP and VP were 1.25, 0.60 and 0.61, respectively. The amount of total oxygenated compounds from carbowax of SCP, CP and VP were 1.37, 0.55 and 0.52, respectively.

The chemical transformations could occur during the distillation of lime oil by subject to high temperatures (95-105 °C) for long periods (6-12 hours) in a very acidic environment (pH 2.2-2.4). The report about lime oil indicated that lime oil suffered deep modifications in its composition : neral, geranial and sabinene almost disappeared; α- and β-pinene reacted to different extent (β-pinene could react approximately 10 times faster than α-pinene to form 1,4- and 1,8-cineole, terpinolene, α-terpineol and *p*-cymene).[4] Although, the essential oils extraction for SCP, CP and VP were the methods which avoided the conditions that might cause chemical transformation, (temperature lower than 95 °C, 3 hours extraction time and without the acidic condition), a little amount of chemical transformated compounds still happened such as: (*Z*)-sabinene hydrate and *p*-cymene. The (*Z*)-sabinene hydrate was detected in SCP, CP and VP less than 0.01% by DB-5 capillary column. The amount of *p*-cymene which was found in SCP and VP were 0.04 and 0.01%, respectively, by carbowax capillary column.

There were some chemicals that could not be detected by polar column and some could not be detected by nonpolar column. Therefore, using of two different column polarities could present some of different chemical compounds that were determined only in each column. The chemical components of peel oils that were detected only in DB-5 were γ-terpinene, (*Z*)-limonene oxide, (*E*)-limonene oxide, citronellal, γ-terpineol, *n*-decanal, (*Z*)-(+)-carveol, indole, α-terpinyl acetate, neryl acetate, α-copaene, β-(-)-cubebene, dodecanal, β-copaene, α-(-)-selinene, δ-(+)-cadinene, germacrene D-4-ol, tetradecanal, (*Z*, *Z*)-farnesol and (*E*, *E*)-farnesol. The chemical components of peel oils that were detected only in carbowax were δ-

elemene, β-(-)-elemene, linayl acetate, β-(+)-selinene, citronellol and (+)-spathulenol. SCP contained higher amount of α-terpineol, neral and geranial than the other oils, while CP had β-(+)-pinene in higher amount than the other oils. In addition, SCP, CP and VP showed close amount of sabinene and α-(-)-pinene. These results suggested that SCO_2 had extraction capacity stronger than the other methods. SCO_2 gave less of deterioration of essential oil components. Since SCO_2 method avoided factors that caused chemical transformations. The VP really showed higher deterioration of some volatile components than CP.

Table 10 Classification of the chemical compositions of *C. maxima* peel and flower essential oils and neroli

Compounds group	Compounds name
Acyclic monoterpene hydrocarbons	(*Z*)-β-ocimene
	(*E*)-β-ocimene
	myrcene
Cyclic monoterpene hydrocarbons	*p*-cymene
	α -(-)-pinene
	β-(+)-pinene
	α -phellandrene
	terpinolene
	α -thujene
	camphene
	γ-terpinene
	R-(+)-limonene
	sabinene
Acyclic oxygenated monoterpenes	(*E*,*E*)-farnesol
	(*E*,*Z*)-farnesol
	(*Z*, *E*)-farnesol
	(*Z*,*Z*)-farnesol
	citronellol
	linalool
	nerol
	citronellal
	geranial
	neral
	dihydrolinalyl acetate
	neryl acetate
	geranyl acetate
	geranyl formate
	linalyl acetate

Table 10 Classification of the chemical compositions of *C. maxima* peel and flower essential oils and neroli (continued)

Compounds group	Compounds name
Cyclic oxygenated monoterpenes	α -terpineol
	(*Z*)-(+)-carveol
	(*E*)-(+)-carveol
	γ -terpineol
	geraniol
	terpinen-4-ol
	perilla aldehyde
	α -terpinyl acetate
	(*Z*)-limonene oxide
	(*E*)-limonene oxide
	(*Z*)-(-)-linalool oxide
	(*E*)-(+)-linalool oxide
	(*E*)-sabinene hydrate
Acyclic sesquiterpene hydrocarbons	(*E*,*E*)- α –farnesene
Cyclic sesquiterpene hydrocarbons	α -copaene
	β-copaene
	α -(-)-cubebene
	β-(-)-cubebene
	α -humulene
	α -(-)-selinene
	β-(+)-selinene
	δ-(+)-cadinene
	β-(-)-elemene
	δ-elemene
	γ -(+)-elemene
	bicyclogermacrene
	germacrene B
	germacrene D
	β-caryophyllene

Table 10 Classification of the chemical compositions of *C. maxima* peel and flower essential oils and neroli (continued)

Compounds group	Compounds name
Acyclic oxygenated sesquiterpenes	(*E*)-nerolidol
Cyclic oxygenated sesquiterpenes	germacrene D-4-ol
	(+)-spathulenol
	(+)-nootkatone
	caryophyllene oxide
Aromatic compounds	phenyl ethyl alcohol
	methyl anthanilate
	methyl benzoate
	2-(formylamino)-bezoate
	indole
Long chain hydrocarbons	3,7-dimethyl-1,5-octadien-3,7-diol
	3,7-dimethyl-2,6-octadien-1-ol
	dodecanal
	n-decanal
	tetradecanal
Miscellaneous	unknowns

Table 11 The chemical compositions of *C. maxima* peel and flower essential oils and neroli

Chemical compounds	% Area				
	SCP	CP	VP	SC-f	neroli
DB-5					
Acyclic monoterpene hydrocarbons	1.77	1.94	1.87	4.68	3.62
Cyclic monoterpene hydrocarbons	95.47	97.28	96.98	89.61	19.99
Acyclic oxygenated monoterpenes	0.76	0.23	0.38	3.62	64.70
Cyclic oxygenated monoterpenes	0.25	0.34	0.19	0.11	3.37
Acyclic sesquiterpene hydrocarbons	0.03	0.01	-	0.04	0.01
Cyclic sesquiterpene hydrocarbons	1.40	0.13	0.53	1.18	0.58
Acyclic oxygenated sesquiterpenes	0.03	0.02	-	0.59	0.03
Cyclic oxygenated sesquiterpenes	0.09	-	-	0.59	0.03
Aromatic compounds	0.05	0.01	0.01	0.09	7.34
Long chain hydrocarbons	0.11	0.01	0.03	0.04	0.33
Miscellaneous	0.04	0.04	0.01	0.01	-
Carbowax					
Acyclic monoterpene hydrocarbons	2.33	2.03	1.35	1.97	2.83
Cyclic monoterpene hydrocarbons	94.77	97.21	97.52	90.64	18.92
Acyclic oxygenated monoterpenes	0.79	0.44	0.39	4.90	59.00
Cyclic oxygenated monoterpenes	0.39	0.14	0.13	0.07	6.04
Acyclic sesquiterpene hydrocarbons	-	-	-	-	**could not calculated**
Cyclic sesquiterpene hydrocarbons	1.56	0.18	0.60	1.14	0.58
Acyclic oxygenated sesquiterpenes	0.01	0.01	-	0.77	0.04
Cyclic oxygenated sesquiterpenes	0.16	-	-	0.43	0.05
Aromatic compounds	0.02	-	-	0.06	5.41
Long chain hydrocarbons	-	-	-	0.01	1.81
Miscellaneous	-	-	-	-	-

Table 12 Qualitative and quantitative analysis of *C. maxima* flower essential oil (SC-f) and *C. aurantium* var *amara* (neroli) analysed by using DB-5 capillary column

Peak no.[a]	KI		Components[c]	% Area	
	Reference[b]	Sample		SC-f	neroli
1	930	933	α -thujene	trace	0.02
2	939	941	α -(-)-pinene	0.57	2.91
3	955	959	camphene	trace	0.12
4	975	979	sabinene	0.56	0.18
5	979	983	β-(+)-pinene	0.88	0.96
6	991	994	myrcene	1.77	1.40
7	1003	1008	α -phellandrene	1.35	0.57
8	1029	1025	*R*-(+)-limonene	86.20	14.99
9	1037	1045	(*Z*)-β-ocimene	0.06	0.86
10	1050	1055	(*E*)-β-ocimene	2.85	1.37
11	1060	1065	γ-terpinene	trace	0.02
12	1070	1072	(*Z*)-sabinene hydrate	trace	no
13	1073	1075	(*E*)-(+)-linalool oxide	no	0.05
14	1089	1091	terpinolene	0.22	0.22
15	1091	1095	methyl benzoate	no	0.98
16	1097	1096	linalool	0.38	22.12
17	1107	1117	phenyl ethyl alcohol	no	1.60
18	1137	1138	(*Z*)-limonene oxide	0.01	0.05
19	1142	1148	(*E*)-limonene oxide	trace	0.02
20	1153	1156	citronellal	0.03	0.03
21	1189	1192	α -terpineol	0.03	3.14
22	1202	1205	*n*-decanal	0.02	0.32
23	1230	1230	nerol	0.19	3.19
24	1238	1244	neral	0.73	0.04
25	1253	1257	geraniol	0.05	0.02
26	1257	1266	linalyl acetate	no	31.47
27	1267	1273	geranial	1.17	0.09
28	1275	1281	dihydrolinalyl acetate	trace	0.01
29	1291	1292	indole	0.03	0.02
30	1298	1302	geranyl formate	no	0.06
31	1337	1343	methyl antranilate	0.06	4.55
32	1349	1353	α -terpinyl acetate	no	0.09
33	1362	1366	neryl acetate	0.10	2.96
34	1377	1382	α -copaene	0.01	0.01
35	1384	1384	geranyl acetate	0.12	4.81
36	1388	1387	β-(-)-cubebene	0.05	no
37	1391	1396	β-(-)-elemene	no	0.02
38	1409	1410	dodecanal	0.01	0.01
39	Ms	1412	unknown from lime oil	trace	no
40	1419	1426	β-caryophyllene	0.23	0.34
41	1437	1435	β-copaene	0.01	trace
42	1455	1460	α -humulene	0.02	0.04
43	1485	1488	germacrene D	0.45	0.01
44	1498	1497	α -(-)-selinene	0.01	0.01
45	1500	1501	bicycolgermacrene	0.30	0.08

Table 12 Qualitative and quantitative analysis of *C. maxima* flower essential oil (SC-f) and *C. aurantium* var *amara* (neroli) analysed by using DB-5 capillary column (continued)

Peak no.[a]	KI		Components[c]	% Area	
	Reference[b]	Sample		SC-f	neroli
46	1506	1510	(*E*, *E*)-α -farnesene	0.04	trace
47	1523	1529	δ-(+)-cadinene	0.02	0.02
48	Ms	1557	2-(formylamino)-bezoate	no	0.31
49	1561	1558	germacrene B	trace	no
50	1563	1567	(*E*)-nerolidol	0.59	0.03
51	1576	1582	germacrene D-4-ol	0.03	no
52	1578	1583	(+)-spathulenol	no	0.04
53	1583	1588	caryophyllene oxide	no	0.03
54	1613	1614	tetradecanal	trace	no
55	1701	1700	(*Z*, *E*)-farnesol	0.05	no
56	1718	1719	(*Z*, *Z*)-farnesol	0.06	no
57	1725	1737	(*E*, *E*)-farnesol	0.79	no
58	1746	1755	(*E*, *Z*)-farnesol	trace	no
59	1760	1766	benzyl benzoate	no	0.40
60	1807	1810	(+)-nootkatone	0.01	no

[a]Peak numbers refer to Figure 30 and 31

[b]KI from R. Adams[85]

[c]Peak identifications are based on MS comparison with file spectra and relative retention time(KI)

trace : less than 0.01%

SC-f : essential oil of *C. maxima* flower by supercritical carbon dioxide extraction

neroli : essential oil of *C. aurantium* var. *amara* flower by solvent extraction

Ms : identification from mass spectra of willey database library and/or R. Adam

no : could not detected

Table 13 Qualitative and quantitative analysis of *C. maxima* flower essential oil (SC-f) and *C. aurantium* var *amara* (neroli) analysed by using carbowax capillary column

Peak no.[a]	KI		Components[c]	% Area	
	Reference	Sample		SC-f	neroli
1	1036[d]	1029	α -(-)-pinene	0.63	2.96
2	1078[d]	1072	camphene	0.01	0.11
3	1120[d]	1110	β-(+)-pinene	0.89	0.91
4	1130[e]	1120	sabinene	0.68	0.20
5	1156[d]	1154	myrcene	1.82	1.53
6	1173[d]	1163	α -phellandrene	1.61	0.03
7	1210[d]	1211	*R*-(+)-limonene	86.76	14.50
8	1228[d]	1223	(*Z*)-β-ocimene	0.10	0.39
9	1250[d]	1244	(*E*)-β-ocimene	0.05	0.91
10	1272[e]	1261	*p*-cymene	0.04	0.05
11	1279[d]	1275	terpinolene	0.02	0.16
12	1451[d]	1449	(*E*)-(+)-linalool oxide	no	0.09
13	1423[d]	1478	(*Z*)-(-)-linalool oxide	no	0.05
14	1484[e]	1496	*n*-decanal	no	0.02
15	1555[d]	1546	linalool	0.42	
16	1591[d]	1558	β-(-)-elemene	0.01	49.50[combinded]
17	1569[d]	1561	linalyl acetate	no	
18	1562[d]	1587	β-caryophyllene	0.28	0.39
19	1600[e]	1607	methyl benzoate	no	0.97
20	1601[d]	1611	terpinen-4-ol	no	0.05
21	Ms	1637	carbitol	no	0.55
22	1672[d]	1660	α -humulene	0.04	0.04
23	1680[d]	1683	neral	0.82	0.05
24	1712[e]	1698	germacrene-D	0.56	0.11
25	1699[d]	1712	neryl acetate	0.27	3.08
26	1717[d]	1719	geranyl formate	no	3.20
27	1738[d]	1725	bicyclogermacrene	0.23	no
28	1730[d]	1736	geranial	1.30	0.06
29	1754[d]	1745	geranyl acetate	0.18	
30	1756[d]	1752	(*E*, *E*)-α -farnesene	no	5.31 [combinded]
31	1765[d]	1775	citronellol	no	0.03
32	1818[d]	1787	perilla aldehyde	0.01	trace
33	1808[d]	1806	nerol	0.20	3.09
34	1864[d]	1820	germacrene B	0.02	3.09
35	1842[d]	1856	geraniol	0.06	5.85
36	Ms	1868	3,7-dimethyl-2,6-octadien-1-ol	no	0.10
37	1967[d]	1922	tetradecanal	0.01	1.43
38	Ms	1974	3,7-dimethyl-1,5-octadien-3,7-diol	no	0.10
39	2044[d]	2039	(*E*)-nerolidol	0.77	0.04

Table 13 Qualitative and quantitative analysis of *C. maxima* flower essential oil (SC-f) and *C. aurantium* var *amara* (neroli) analysed by using carbowax capillary column (continued)

Peak no.[a]	KI		Components[c]	% Area	
	Reference	Sample		SC-f	neroli
40	2153[d]	2144	(+)-spathulenol	no	0.05
41	2260[e]	2231	methyl anthanilate	0.06	3.89
42	2250[d]	2254	(+)-nootkatone	0.43	no
43	Ms	2274	(*Z*, *E*)-farnesol	0.42	no
44	Ms	2325	(*Z*, *Z*)-farnesol	0.09	no
45	2371[g]	2336	(*E*, *E*)-farnesol	0.08	no
46	Ms	2411	(*E*, *Z*)-farnesol	1.13	no

[a]Peak numbers refer to Figure 32 and 33

[c]Peak identifications are based on MS comparison with file spectra and relative retention time (KI)

[d]KI from N.W. Davies[87]

[e]KI from T. Acree and H. Arn[88]

[f]KI from S.M. Njoroge and other[89]

[g]KI from A.M. El-Sayed[90]

Ms : identified from mass spectrums compare with standard compound from R. Adam [85]

trace : less than 0.01%

SC-f : essential oil of *C. maxima* flower by supercritical carbon dioxide extraction

neroli : essential oil of *C. aurantium* var. *amara* flower by solvent extraction

[combinded] relative area of merged peaks

no : could not detected

The elution profile of *C. maxima* flower oil (SC-f, spectra 12 and 14) and neroli (13 and 15) using DB-5 and carbowax capillary column, respectively. The identification and the relative area of chemicals of *C. maxima* flower oil and neroli was shown in Table 12 and 13. For neroli analysis using carbowax, there were 2 merged peaks happen at peak numbers 15 to 17 and peak numbers 29 to 30. The first merged peak contained linalool, β-(-)-elemene and linalyl acetate. The second merged peak contained (*E,E*)-α-farnesene and geranyl acetate. This conclusion was confirmed by peaks and % relative areas of those compounds in neroli that were separated by DB-5 column.

The analysed data of DB-5 column showed that SC-f and neroli had 49 constituents but some compounds were different. The compounds that appeared in both SC-f and neroli were *R*-(+)-limonene, linalool, α-(-)-, β-(+)-pinene, sabinene, myrcene, α-phellandrene, (*Z*)-β-ocimene, (*E*)-β-ocimene, γ-terpinene, terpinolene, citronellal, α-tepineol, nerol, neral, geraniol, geranial, indole, methyl anthranilate, neryl acetate, geranyl acetate, dodecanal, β-caryophyllene, α-humulene, germacrene D, α-(-)-selinene, bicyclogermacrene, (*E,E*)-α-farnesene, δ-(+)-cadinene, (*E*)-nerolidol, etc. However, their relative peak areas were different. When the data were categorized in Table 11, the results showed that neroli contained more various type of oxygenated compounds than SC-f, especially oxygenated cyclic monoterpenes, oxygenated acyclic monoterpenes and other oxygenated compounds such as neryl acetate (2.96, 0.10%), geranyl acetate (4.81, 0.12%), methyl anthranilate (4.55, 0.06%), linalool (22.12, 0.38%), nerol (3.19, 0.19%), [percent in parenthesis means % relative amount of those compound belongs to neroli and SC-f, repectively]. While the SC-f contained more terpene hydrocarbons than neroli (90.83, 20.55%), especially, *R*-(+)-limonene (86.20, 14.99%), [percent in parenthesis means % relative amount of those compound belongs to SC-f and neroli, repectively]. The compounds that were found only in neroli when detected with DB-5 and carbowax were methyl benzoate, benzyl benzoate, 2-(formylamine)-benzoate, (+)-spathulenol, caryophyllene oxide, linalyl acetate and phenyl ethyl alcohol. On the other hand, the compounds that were found only in SC-f when detected with DB-5 and carbowax were (*Z*)-sabinene hydrate, β-(-)-cubebene, gernacrene D-4-ol, farnesol and (+)-nootkatone. These different results may cause by the different species and extraction methods of the raw

materials. By using solvent extraction, neroli still contained the solvent residue, phenyl ethyl alcohol, and other additive substances, benzoate compounds.

(+)-Nootkatone is the characteristic aroma compound of grapefruit peel oil, which genetic relatively close to *C. maxima*.[1, 92] (+)-Nootkatone was also found in the *C. maxima* flower by SCO_2 extraction but was not found in steam distillation flower oil from Vietnam.[60] There were some literatures reported the appearance of some isomer of farnesol in neroli oil derived from bitter orange flower.[5] However, in this study, the farnesol could not be detected in neroli oil derived from *C.aurantium* var. *amara*. The GC analysis data of SCP and SC-f showed that, they contained low amount of all isomer of farnesol. As suggestion above, the SCO_2 extraction could extract the higher boiling points components better than the CPM and VSD.

The volatile components of SC-f and neroli that were detected only by DB-5 were γ-terpinene, (*Z*)-sabinene hydrate, phenyl ethyl alcohol, (*Z*)-limonene oxide, (*E*)-limonene oxide, citronellal, α-terpineol, *n*-decanol, dihydrolinayl acetate, indole, α-terpinyl acetate, α-copaene, dodecanal, β-copaene, α-(-)-selinene, δ-(+)-cadinene, 2-(formylamino)-benzoate, germacrene D-4-ol, caryophyllene oxide, (*E*,*E*)-fanesol, (*E*,*Z*)-fanesol and benzyl benzoate.

The volatile component of SC-f and neroli that were detected only by carbowax were *p*-cymene, carbitol, β-citronellol, perilla aldehyde, 3,7-dimethyl-2,6-octadiene-1-ol, 3,7-dimethyl-1,5-octadiene-3,7-diol.

Table 14 Comparison between the *C. maxima* flower essential oil from Vietnam and SC-f

Components	% Amount	
	Vietnam	SC-f
heptanal	< 0.05	no
α -thujene	< 0.05	< 0.01
α -(-)-pinene	0.40	0.57
camphene	< 0.05	< 0.01
sabinene	0.70	0.56
β-pinene	3.40	0.88
myrcene	3.90	1.77
α -phellandrene	1.30	1.35
p-cymene	0.10	0.04
β-phellandrene	< 0.05	no
R-(+)-limonene	18.20	86.20
(*Z*)-ocimene	0.10	0.06
(*E*) -ocimene	4.40	2.85
γ -tepinene	0.10	< 0.01
linalool	16.40	0.38
terpinen-4-ol	< 0.05	no
3-hexenyl butyrate	< 0.05	no
α -terpineol	< 0.05	0.03
nerol	1.10	0.19
neral	0.50	0.73
geraniol	0.60	0.05
geranial	0.50	1.17
methyl anthranilate	0.30	0.06
neryl acetate	< 0.05	0.10
geranyl acetate	< 0.05	0.12
β-caryophyllene	0.40	no
geranyl acetone	0.20	no
nerolidol	29.30	0.50
farnesal	0.30	no
farnesol isomer	16.70	0.95
farnesyl acetate	0.20	no
5-β-H,7-β,10- α -selina-4(14),11-diene	0.30	no
methyl-*N*-formylantranilate	0.10	no
(*Z*)-sabinene hydrate	no	< 0.01
terpinolene	no	0.22
(*Z*)-limonene oxide	no	0.01
(*E*)-limonene oxide	no	< 0.01
citronellal	no	0.03
n-decanal	no	0.02
dihydrolinalyl acetate	no	< 0.01
indole	no	0.03
α -copaene	no	0.01
α -humulene	no	0.02
germacrene D	no	0.45
α -(-)-selinene	no	0.01
bicyclogermacrene	no	0.30

Table 14 Comparison between the *C. maxima* flower essential oil from Vietnam and SC-f (continued)

Components	% relative amount	
	Vietnam	SC-f
(*E, E*)- α -farnesene	no	0.04
δ-(+)-cadinene	no	0.02
germacrene B	no	< 0.01
germacrene D-4-ol	no	0.03
tetradecanal	no	< 0.01
(+)-nootkatone	no	0.01

SC-f : *C. maxima* flower essential oil by SCO_2 extraction

Vietnam : *C. maxima* (J.Burman) flower essential oil by steam distillation from Vietnam

no : could not detected

The comparison between the *C. maxima* flower essential oil from Vietnam and SC-f were shown in Table 14. The SC-f contained the higher % relative amount of *R*-(+)-limonene, geranial, neryl acetate and geranyl acetate while the %relative amount of linalool, farnesol, nerolidol, geraniol, nerol, ocimene, myrcene and β-(+)-pinene were high in the oil from Vietnam. The SC-f also contained the fewer amounts of high boiling components such as (+)-nootkatone, germacrene, tetradecanal, etc. SC-f did not present some components that had in flower oil from Vietnam such as 3-hexenyl burytate, farnesyl acetate, methyl-*N*-formylantranilate, 5-β-H,7-β,10-α-selina-4(14),11-diene, heptanal, β-phellandrene, terpinen-4-ol, β-caryophyllene, geranyl acetone and farnesal. As the result, the difference might be caused by these different variety, extraction methods and the cultivated area of *C. maxima*. The SC-f was extracted by SCO_2 had come from flower of *C. maxima* cultuva. khao-yai, cultivated in Samutsongkarm province of Thailand, and the oil from Vietnam derived from *C. maxima* flower that was extracted by steam distillation and cultivated in Dan Phuong district, Hanoi, Vietnam.

The comparison the percentages of the main components of flower essential oil among SC-f, neroli, *C. maxima* flower oil prepared by steam distillation from Vietnam[23], and bitter orange flower oil (*C. aurantium* L. subsp. *amara*) by steam distillaiton published in British Pharmacopoeia (BP)[13] were shown in Table 15. The amount of most major components of neroli oil were in the acceptable percentage range that were reported in BP. Neroli contained higher amount of methyl anthranilate and linalyl acetate than that report in BP. The reason of these differ may come from the different extraction methods, different cultivated area and extraction skills. SC-f showed the great difference in amount of *R*-(+)-limonene (86.20%) and linalool (0.38%) from the other Citrus flower essential oils. The SC-f and the oil from Vietnam contained lower amount of the oxygenated compounds than both of *C. aurantium* flower oils, such as α-terpineol, neryl acetate, geranyl acetate and methyl anthranilate. Linalyl acetate was not detected in *C. maxima*. Finally, the (*E*)-nerolidol contained the highest amount in the oil from Vietnam.

Table 15 Comparison of main components in Citrus flower essential oil

Components	% relative amount			
	BP	neroli	SC-f	Vietnam
β-pinene	7.00-17.00	0.96	0.88	3.40
limonene	9.00-18.00	14.99	86.20	18.20
linalool	18.00-42.00	22.12	0.38	16.40
linalyl acetate	3.00-16.00	31.47	0.00	0.00
α -terpineol	2.00-7.00	3.14	0.03	< 0.05
neryl acetate	1.00-3.00	2.96	0.10	< 0.05
geranyl acetate	1.50-4.00	4.81	0.12	< 0.05
(*E*)-nerolidol	1.00-9.00	0.03	0.59	29.30
methyl anthranilate	0.10-1.00	4.55	0.06	0.30

BP : the bitter orange flower oil (*C. aurantium* L. subsp. *amara*) by steam distillation and reported in British Pharmacopeias 2002

neroli : *C. aurantium* var. *amara* flower oil by solvent extraction

SC-f : *C. maxima* Merr. flower oil by SCO_2 extraction

Vietnam : *C. maxima* Merr. flower oil by steam distillation from Vietnam

4. Antimicrobial activity

The SCP and SC-f were the oil extract from the different parts of *Citrus maxima* peel and flower respectively, by the same extraction method, SCO_2. Whereas VP and CP were extracted from the peel of *Citrus maxima* in different extraction method, VSD and CPM, respectively. As shown in Table 16, SCP and SC-f had the MIC at 2.5 µl/ml against *S. aureus* while the other oil samples did not active, except the neroli essential oil gave 50% inhibition at the same concentration. All the oil samples could not inhibit *E. coli*, while SCP, SC-f and neroli showed 50% inhibition against *C. albicans* at 2.5 µl/ml. The essential oils of *Citrus maxima* peel and flower from SCO_2 have shown the best activity against gram positive bacteria. The SCP, SC-f and neroli, which could against *S. aureus* and *C. albicans* at 2.5 µl/ml, could not kill the these microorganism.

Table 16 Minimum inhibitory concentrations (MIC) of essential oil from peel, flower of *C. maxima* and neroli

	MIC (µl/ml)		
Essential oils	*S. aureus*	*E. coli*	*C. albicans*
SCP	2.5	>2.5	2.5 (50% inhibition)
VP	>2.5	>2.5	>2.5
CP	>2.5	>2.5	>2.5
SC-f	2.5	>2.5	2.5 (50% inhibition)
neroli	2.5 (50% inhibition)	>2.5	2.5 (50% inhibition)

As the results indicated, the different antimicrobial activity of the essential oil samples may due to their different chemical compositions or variable amount of monoterpenes**,** sesquiterpenes and oxygenated compounds. Most monoterpenes did not possess a wide spectrum of activity at low dose. Sesquiterpenes alcohol and fatty acids exhibited weak antimicrobial properties towards gram negative bacteria. The aldehyde and phenolic compounds were able to inhibit bacteria, molds, dermatophytes and yeasts at low concentrations.[93] In this study, VP and CP have the high percentage of monoterpenes than SCP, SC-f and neroli. The chemical components identification of essential oil showed that the SCO_2 oils contained the amount of oxygenated compound higher than the others, especially aldehyde which was able to inhibit microorganism at low concentrations. In addition, SCO_2 oils also have the fatty acid, wax and other compounds, that could not find in the other extraction methods,[94] which may effect the result of antimicrobial activity tests by reducing the volatile rate of the samples during incubation period. However, both of SCP and SC-f could not kill the microorganism, they showed the bacteriostatic activity against *S. aureus* at 2.5µl/ml.

CHAPTER V
CONCLUSIONS

The major compounds that were detected in SCP, CP and VP essential oils using DB-5 and carbowax capillary column by GC and GC-MS analysis were *R*-(+)-limonene and myrcene. The amount of *R*-(+)-limonene of each oil that was detected by both columns was close to each other. The SCP presented more number of compounds and more oxygenated components than the other oils. Because SCO_2 could extract the high boiling points components better than the other methods. These three extraction methods showed low chemicals transformation, because they still contained high amount of neral, geranial and sabinene, which were sensitive to transform. Comparison with natural oil derived from cold pressing method, the oils derived from SCO_2 and VSD had small amount of the deterioration compounds such as *p*-cymene which might be changed from other chemical compositions[4], and form hydrolysation during the extraction and raw material preparing procedure than oil derived from CPM.

From this study, SCO_2 was the best method that could solve the limitations and weak points of the other Citrus extraction methods. The smell of Citrus oils from SCO_2 exhibited good quality and quite similar to natural oil. Since, SCP and SC-f contained higher amount of oxygenated compounds than CP and VP. SCO_2 were suitable method for peel oil extraction. Since it could extract dried peel and gave high quality oil with smell naturally alike. The use of dried raw materials reduced difficulty about sample preparation, storage and deficiency.

In the present observations, VP indicated quality of oil resemble to CP and SCP, when compared their chemical constituents. The cost of VSD was lower that SCO_2 and less impurities than CPM. Then asset of production and quality of produced oils should be considered. However, this study did not concern to the maximum quantity of oils from different extraction methods. So, the profit and worth of each extraction methods for perfumery and industries should be further study.

The SC-f contained more *R*-(+)-limonene and other terpene hydrocarbons, but less alcoholic compounds than neroli and steam distillation *C. maxima* flower oil from Vietnam. The comparison between amount of chemical constituents of neroli to orange flower oil in British Pharmacopeias 2002 (standard oil) showed that the amount of major constituents of neroli were close to that of standard oil. However, in commercial, some volatile compounds, such as methyl anthranilate and linalyl acetate, might be added into the oil for improving the quality of smell. Moreover, the commercial oil usually found some solvent and additive compound that not found in Citrus natural oil and BP's neroli, such as phenyl ethyl alcohol, methyl benzoate, benzyl benzoate, etc. These added oxygenated compounds may effect the optical rotation of neroli. The standard optical rotation of neroli (from BP) were +1.5 to +11.5, but optical rotation of neroli (commercial) in this study equaled to +0.875. The SC-f of this study showed the main chemical constituents different from the steam distillation of flower oil from Vietnam. These differences may come from the different in extraction method, cultivated areas and variety of plants. The SC-f and the flower oil from Vietnam indicated the distinction of their components from BP's neroli. Although the amount of major compounds in Vietnam flower oil were more close to BP's neroli than SC-f, the SC-f contained type of chemical components more resemble to that of BP's neroli, commercial neroli and previous reported *C. aurantium* flower oil than Vietnam flower oil. These except for linalyl aceate that found only in *C. aurantium* not in *C. maxima*. For fanesol, it was found only in *C. maxima*. From organoleptic test, SC-f had its own characteristic smell. The SC-f could apply for products in perfumery, aromatherapy and flavoring. So, the development of SCO_2 of *C. maxima* flower was very interesting for further study.

For the antimicrobial activities of peel and flower oil of *C. maxima*, the MIC of SCP and SC-f for *S. aureus* inhibition equaled to 2.5 µl/ml. Neroli showed 50% inhibition for *S. aureus* at 2.5 µl/ml and CP and VP did not active. All of the oil samples could not inhibit *E. coli*, while SCP, SC-f and neroli presented 50% inhibition of *C. albicans* at 2.5 µl/ml. Comparison among *C. maxima* peel extraction methods, the SCP indicated the best activity against gram positive bacteria than the other methods.

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APPENDIX

FIGURES

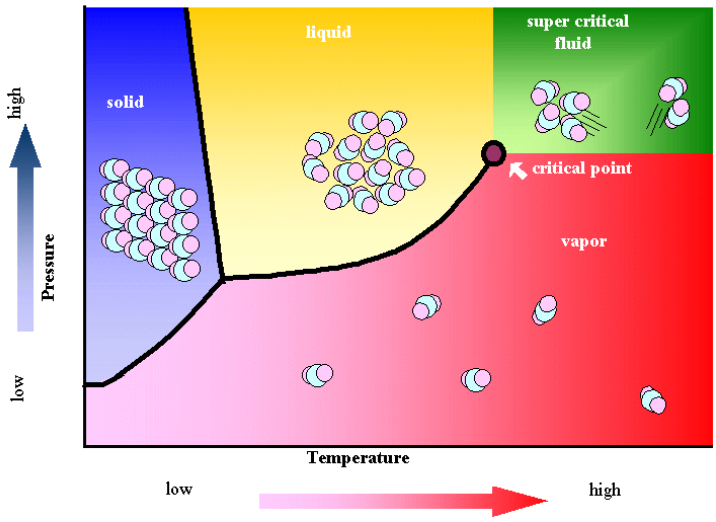


Figure 2 Pressure-temperature phase diagram

A.

B.

Figure 3 *C. maxima* cultivar khao-yai fruit (A) and flower (B) from Bangkontee District, Samutsongkarm Province of Thailand

Figure 4 *C. maxima* dry peel **Figure 5** *C. maxima* fresh peel

Figure 6 The pilot scale of supercritical carbon dioxide extractor at Thai Traditional and Herbal Development Center

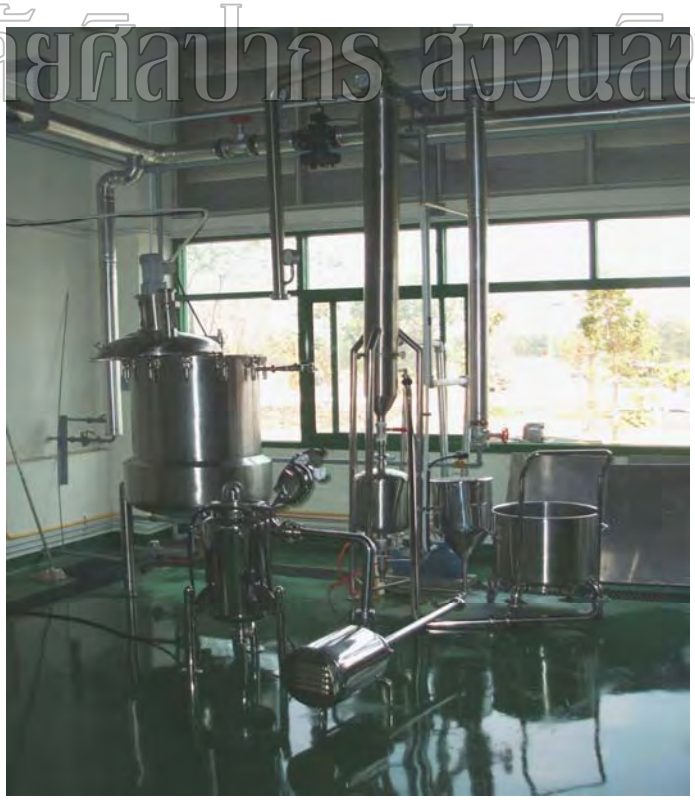


Figure 7 The pilot scale of vacuum steam distillation apparatus at Thai Traditional and Herbal Development Center

Figure 8 SCP : oil derived from *C. maxima* peel, extracted by supercritical carbon dioxide

Figure 9 CP : oil derived from *C. maxima* peel, extracted by cold pressing method

.

Figure 10 VP : oil derived from *C. maxima* peel, extracted by vacuum steam distillation

Figure 11 SC-f : oil derived from *C. maxima* flower, extracted by supercritical carbon dioxide

Figure 12 neroli : oil derived from *C. aurantium* var.*amara*, extracted by organic solvent

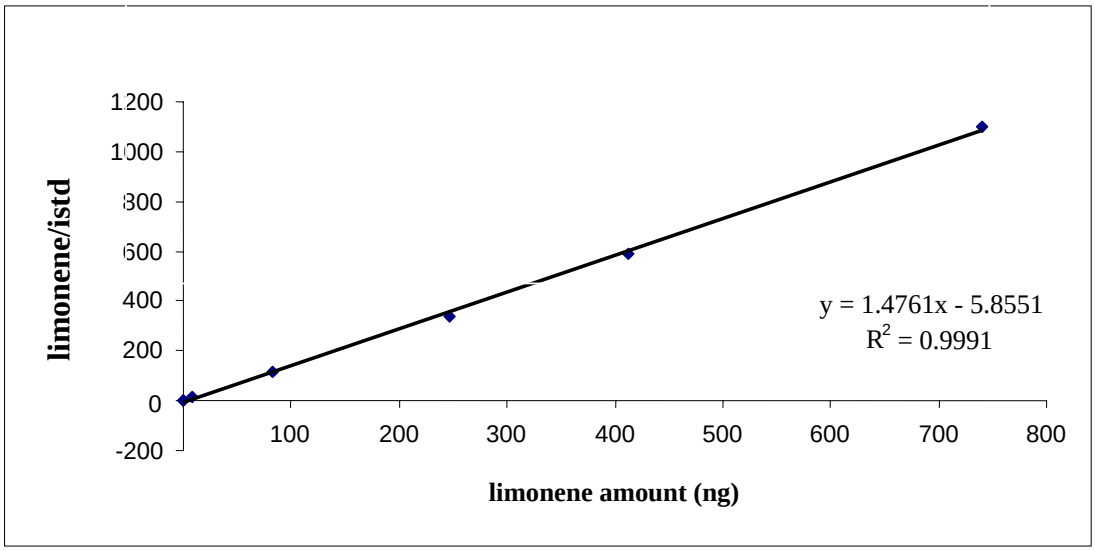


Figure 13 Calibration curve of limonene analysed by using DB-5 capillary column

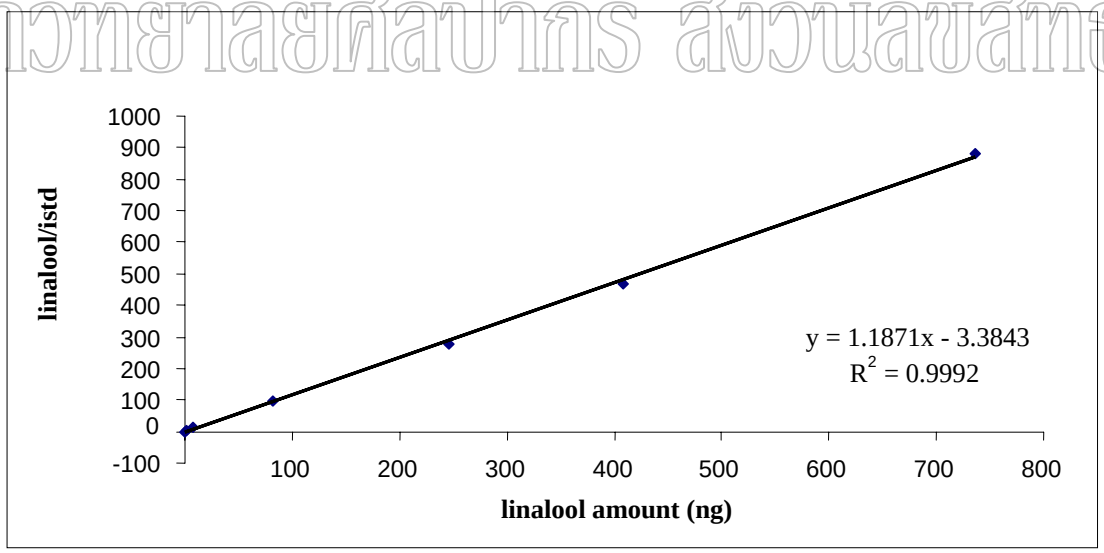


Figure 14 Calibration curve of linalool analysed by using DB-5 capillary column

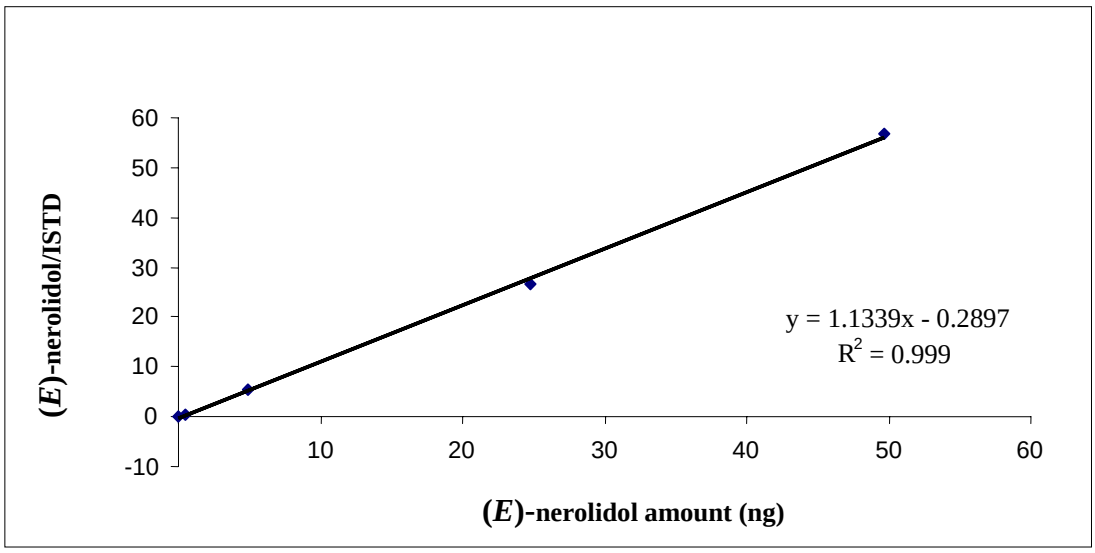


Figure 15 Calibration curve of (*E*)-nerolidol analysed by using DB-5 capillary column

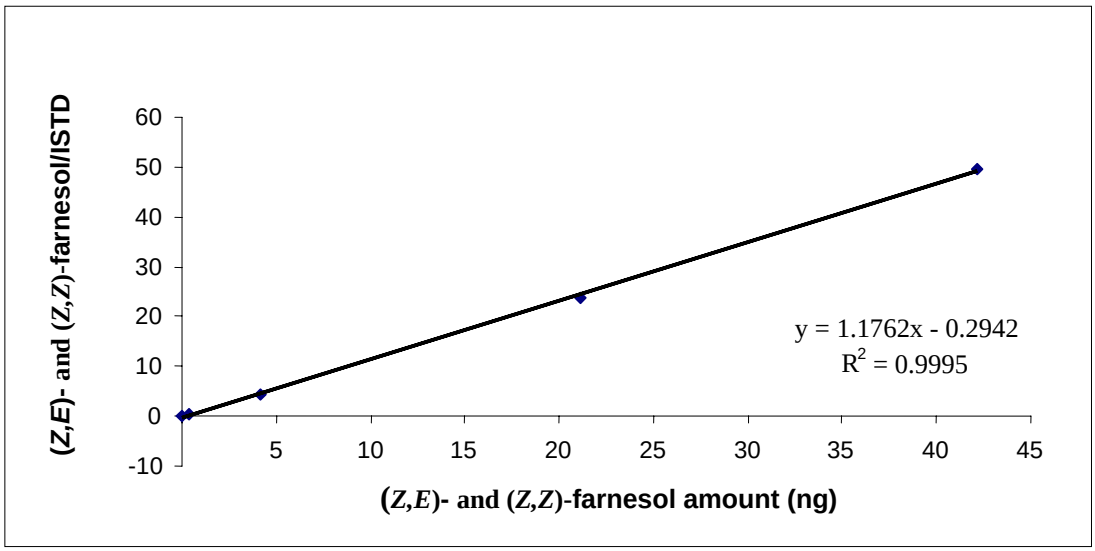


Figure 16 Calibration curve of (*Z*,*E*)- and (*Z*,*Z*)-farnesol analysed by using DB-5 capillary column

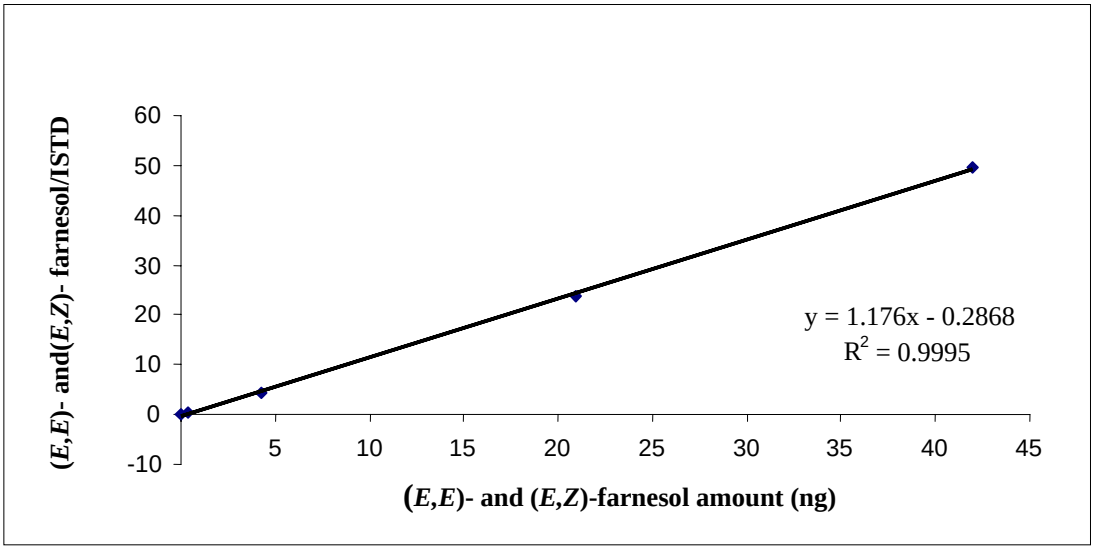


Figure 17 Calibration curve of (*E*,*E*)- and (*E*,*Z*)-farnesol analysed by using DB-5 capillary column

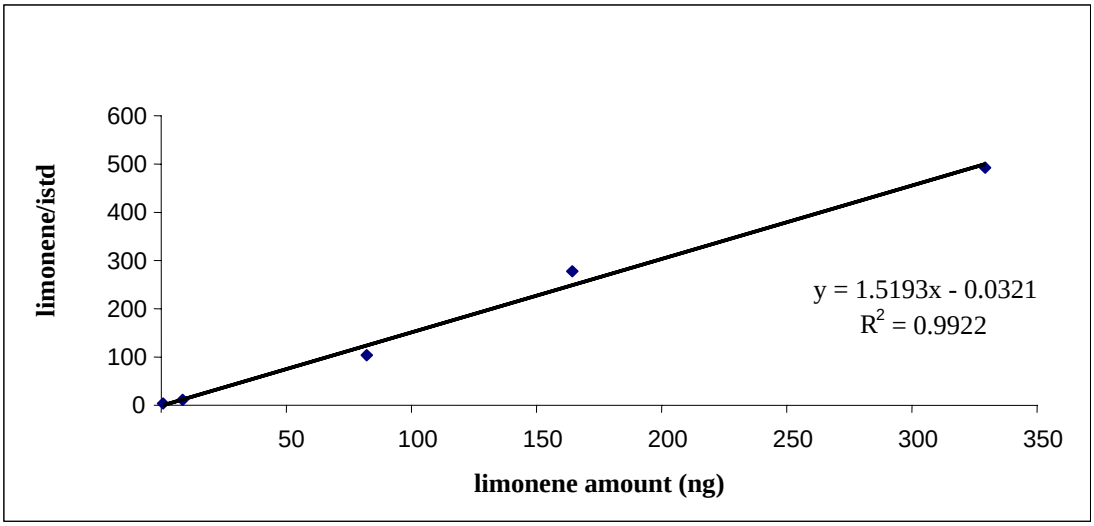


Figure 18 Calibration curve of limonene analysed by using carbowax capillary column

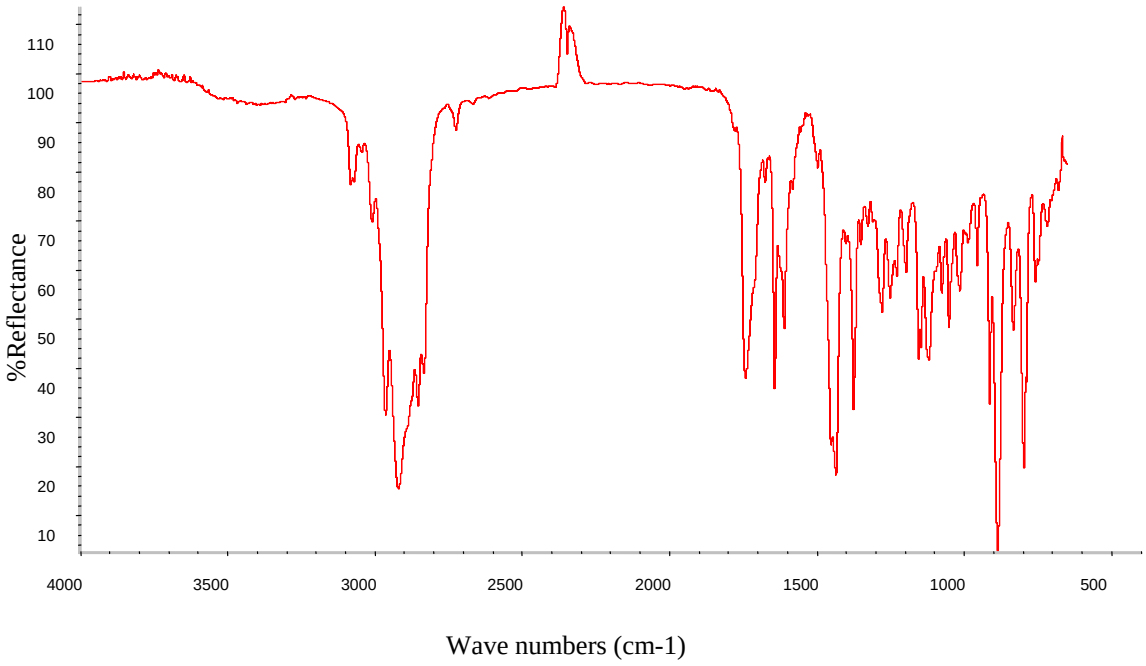


Figure 19 IR spectrum of essential oil derived from *C. maxima* peel, extracted by supercritical carbon dioxide : SCP

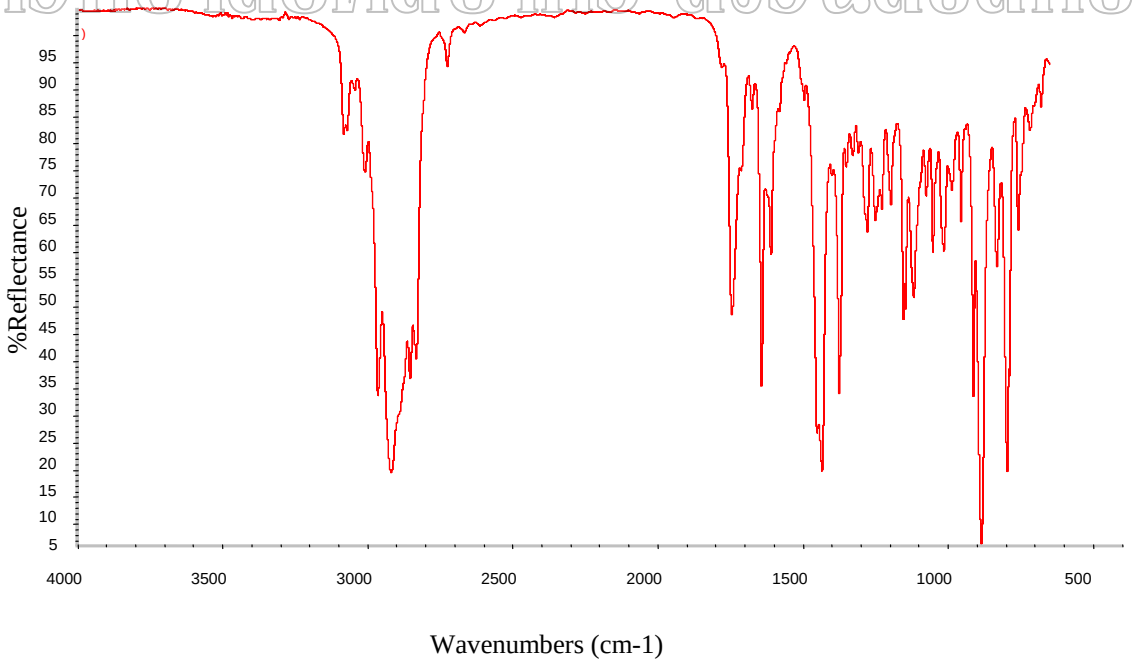


Figure 20 IR spectrum of essential oil derived from *C. maxima* peel, extracted by cold pressing method : CP

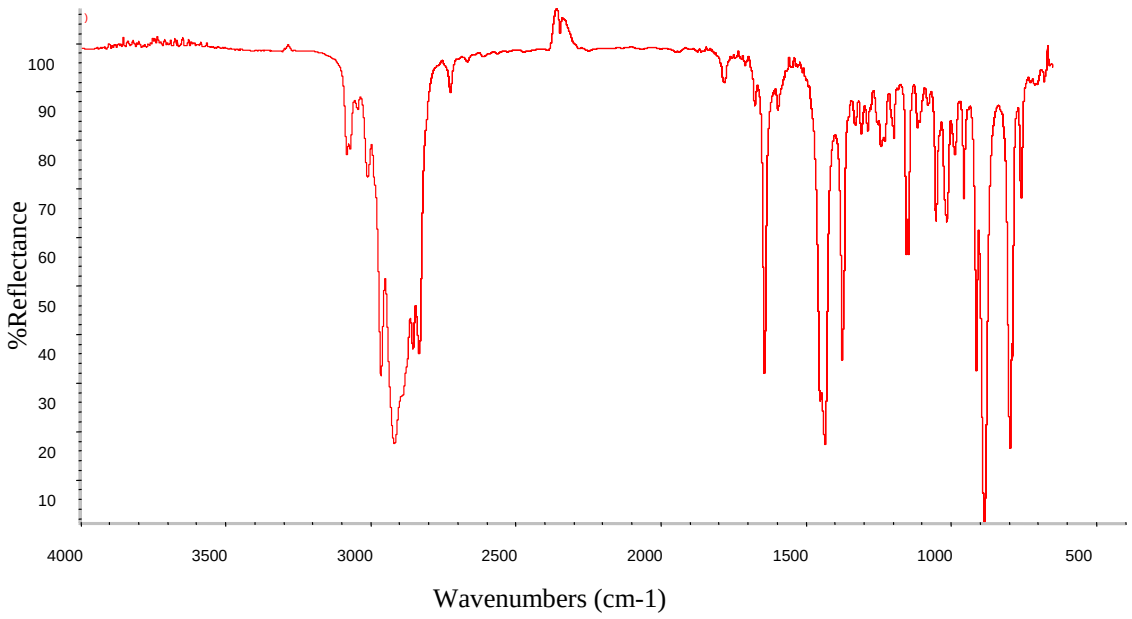


Figure 21 IR spectrum of essential oil derived from *C. maxima* peel, extracted by steam distillation : VP

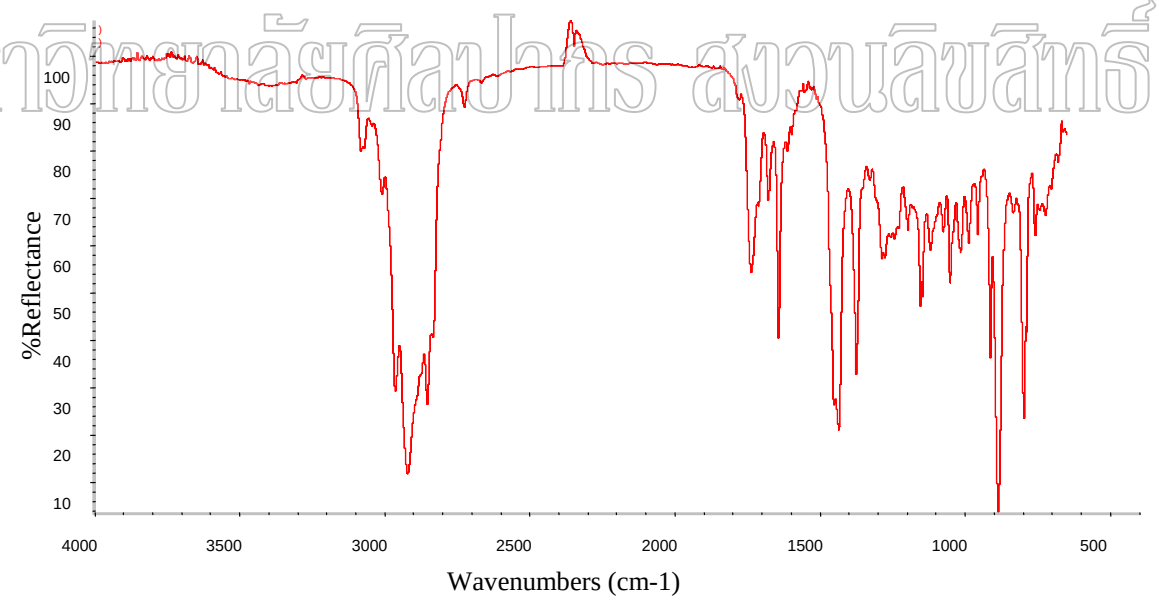


Figure 22 IR spectrum of essential oil derived from *C. maxima* flower, extracted by supercritical carbon dioxide : SC-f

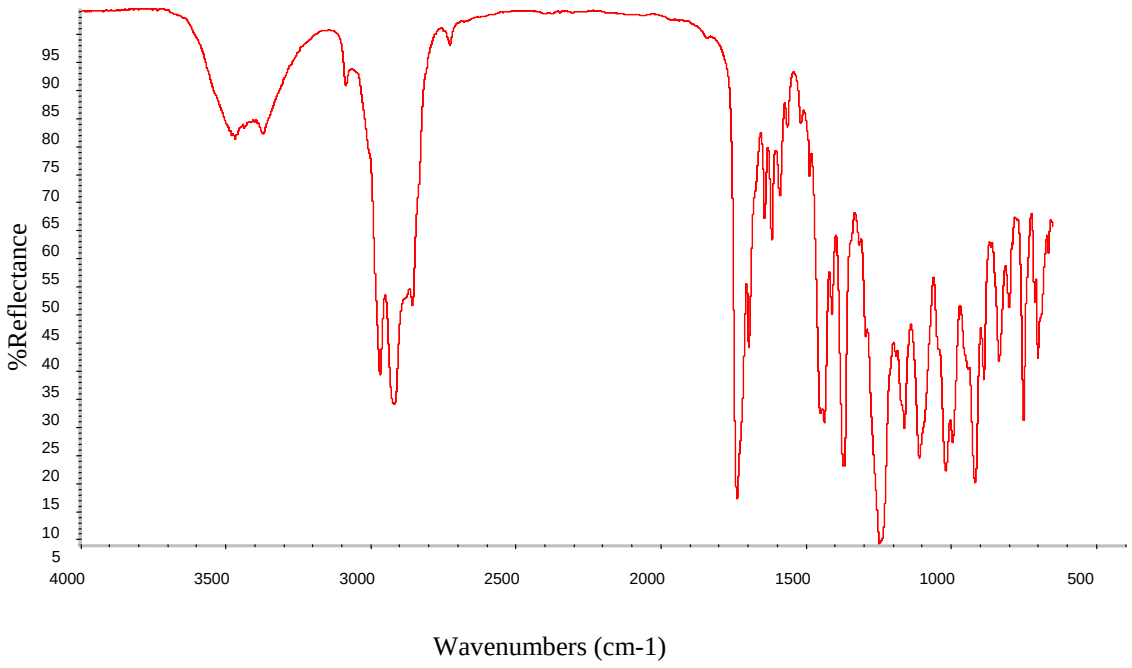


Figure 23 IR spectrum of essential oil derived from *C. aurantium* var. *amara*, extracted by organic solvent : neroli

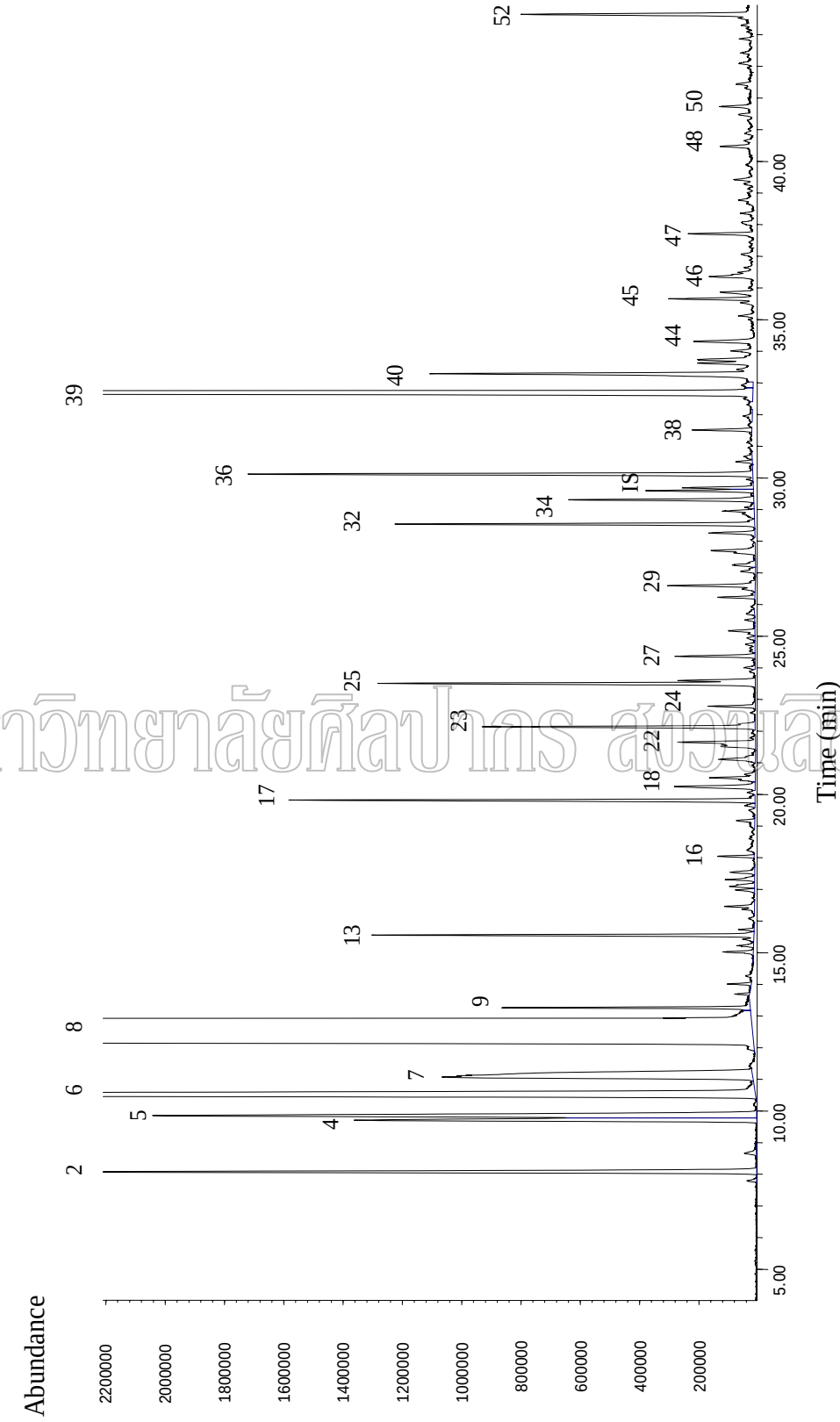


Figure 24 GC chromatogram of *C.maxima* peel essential oil from supercritical carbon dioxide extraction (SCP) analysed by using DB-5 column, IS : internal standard (tetradecane)

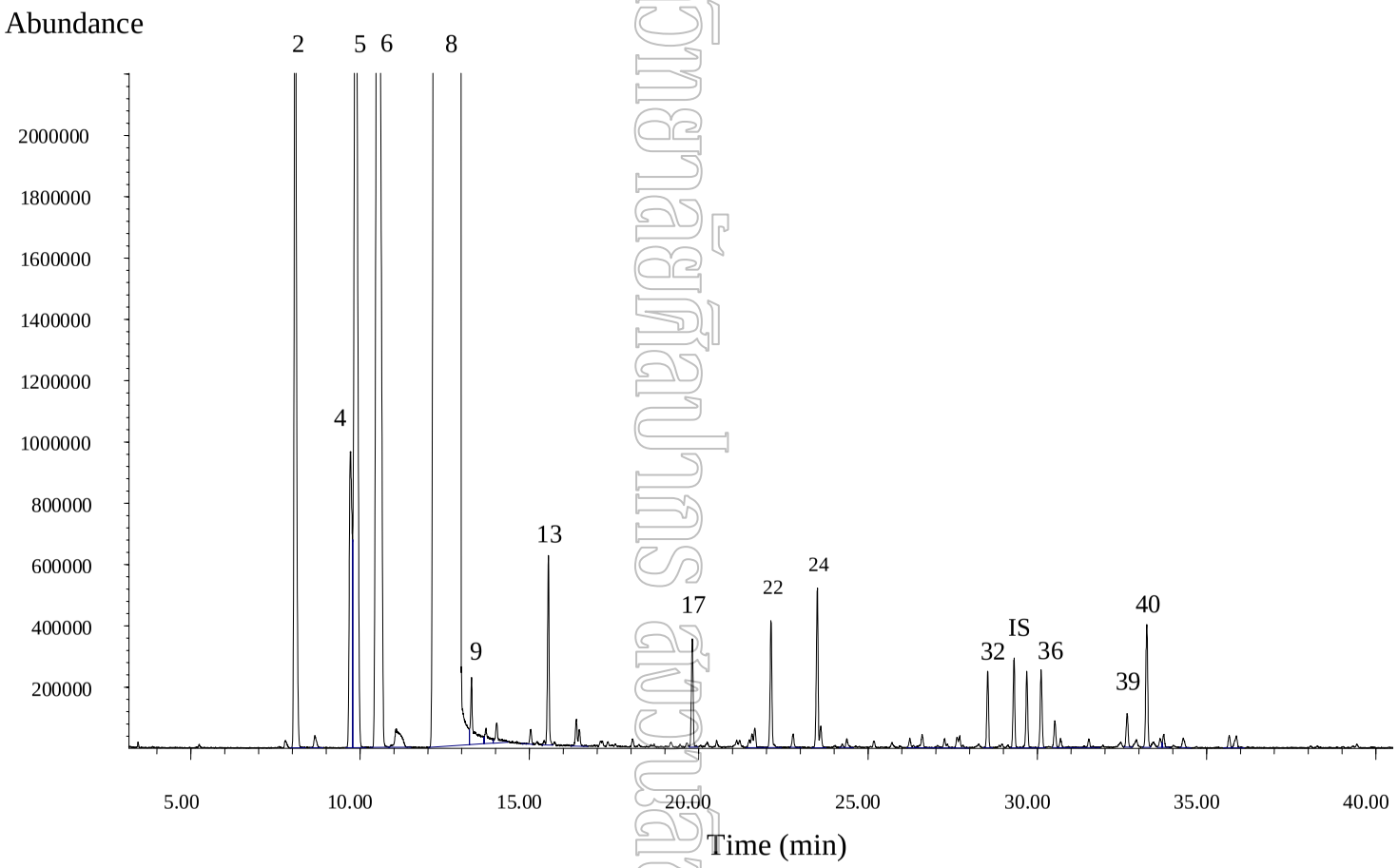


Figure 25 GC chromatogram of *C.maxima* peel essential oil from cold pressing method (CP) analysed by using DB-5 column,
IS : internal standard (tetradecane)

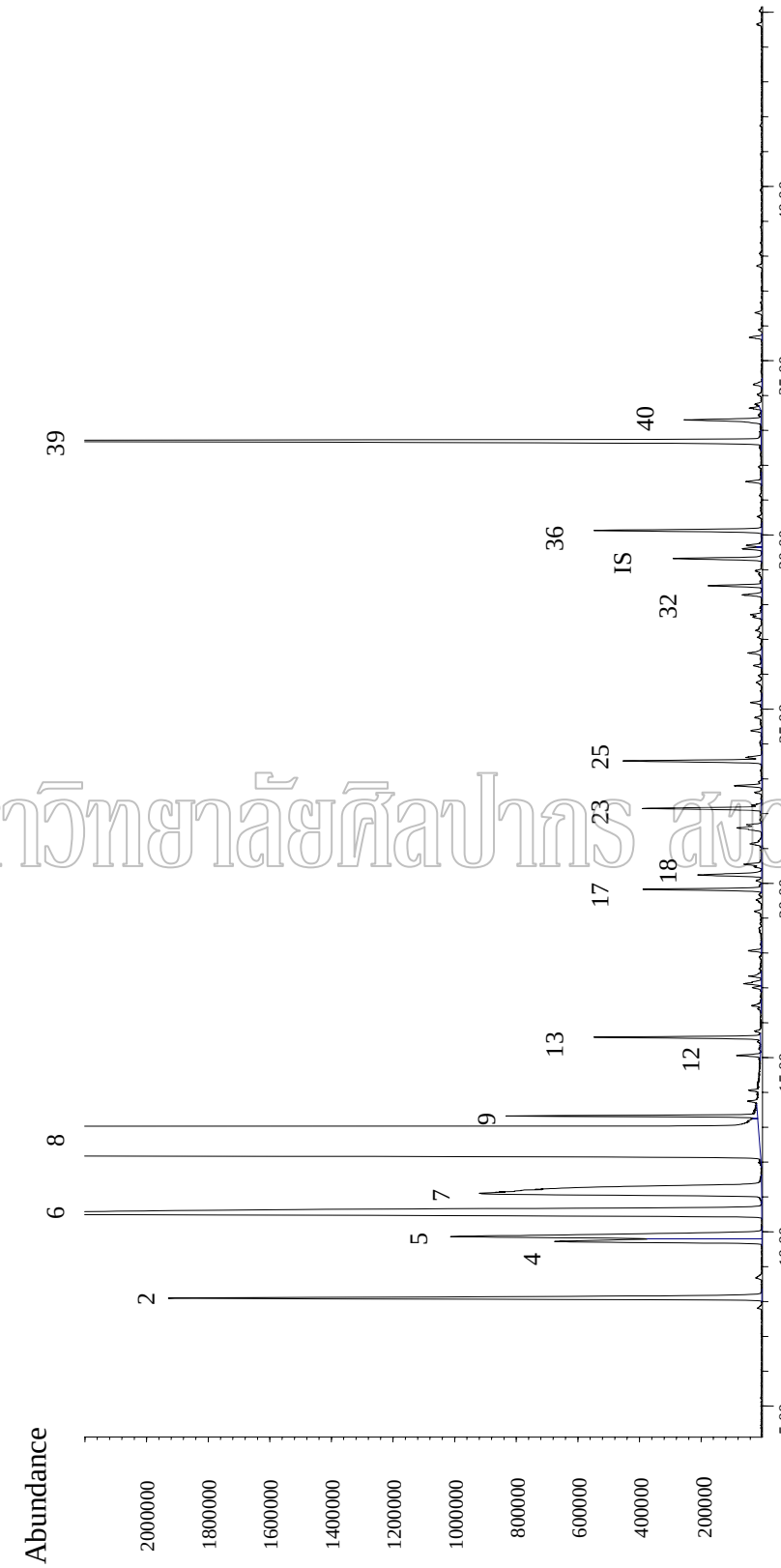


Figure 26 GC chromatogram of *C.maxima* peel essential oil from vacuum steam distillation (VP) analysed by using DB-5 column, IS : internal standard (tetradecane)

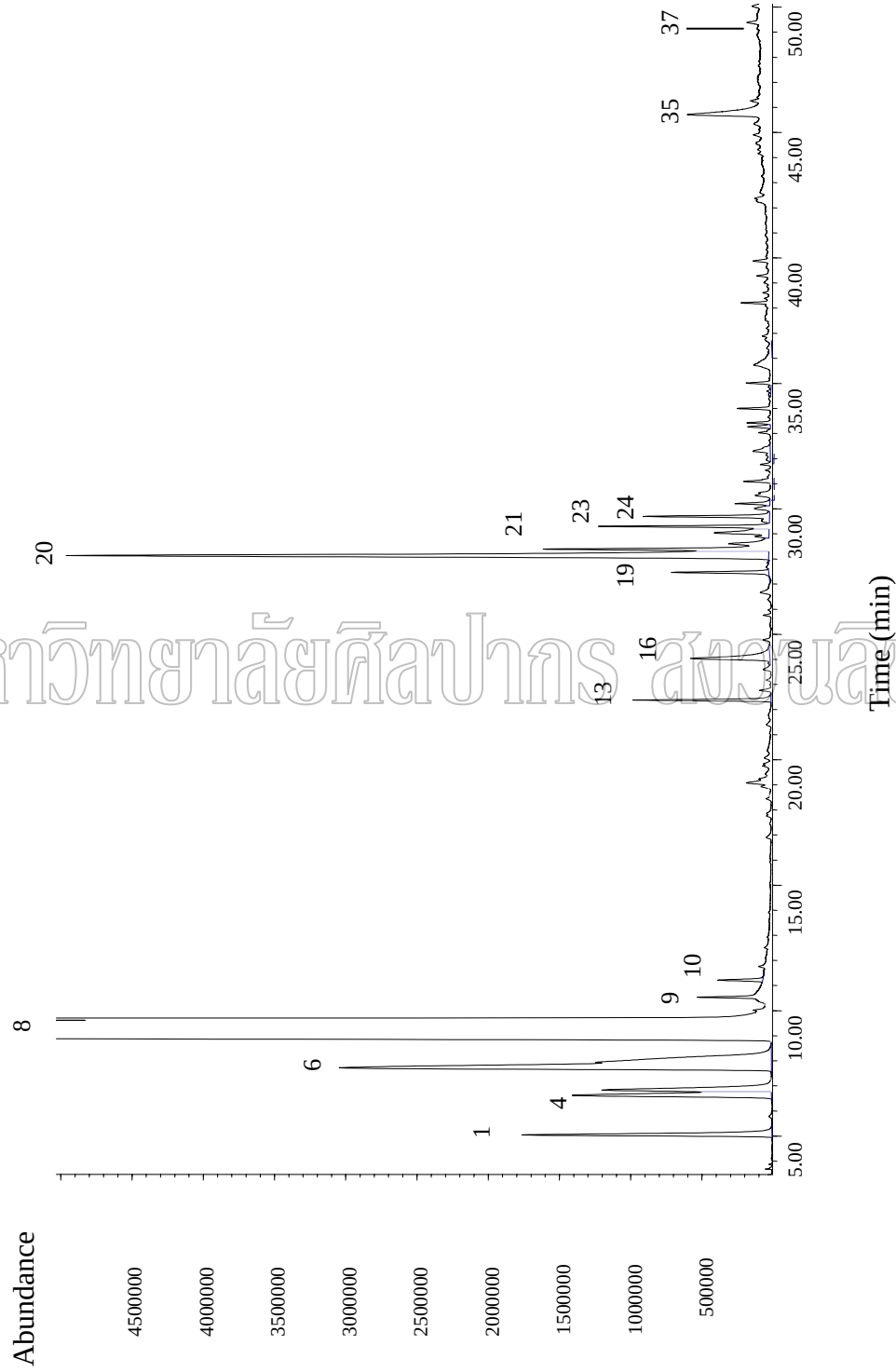


Figure 27 GC chromatogram of *C.maxima* peel essential oil from supercritical carbon dioxide extraction (SCP) analysed by using carbowax column

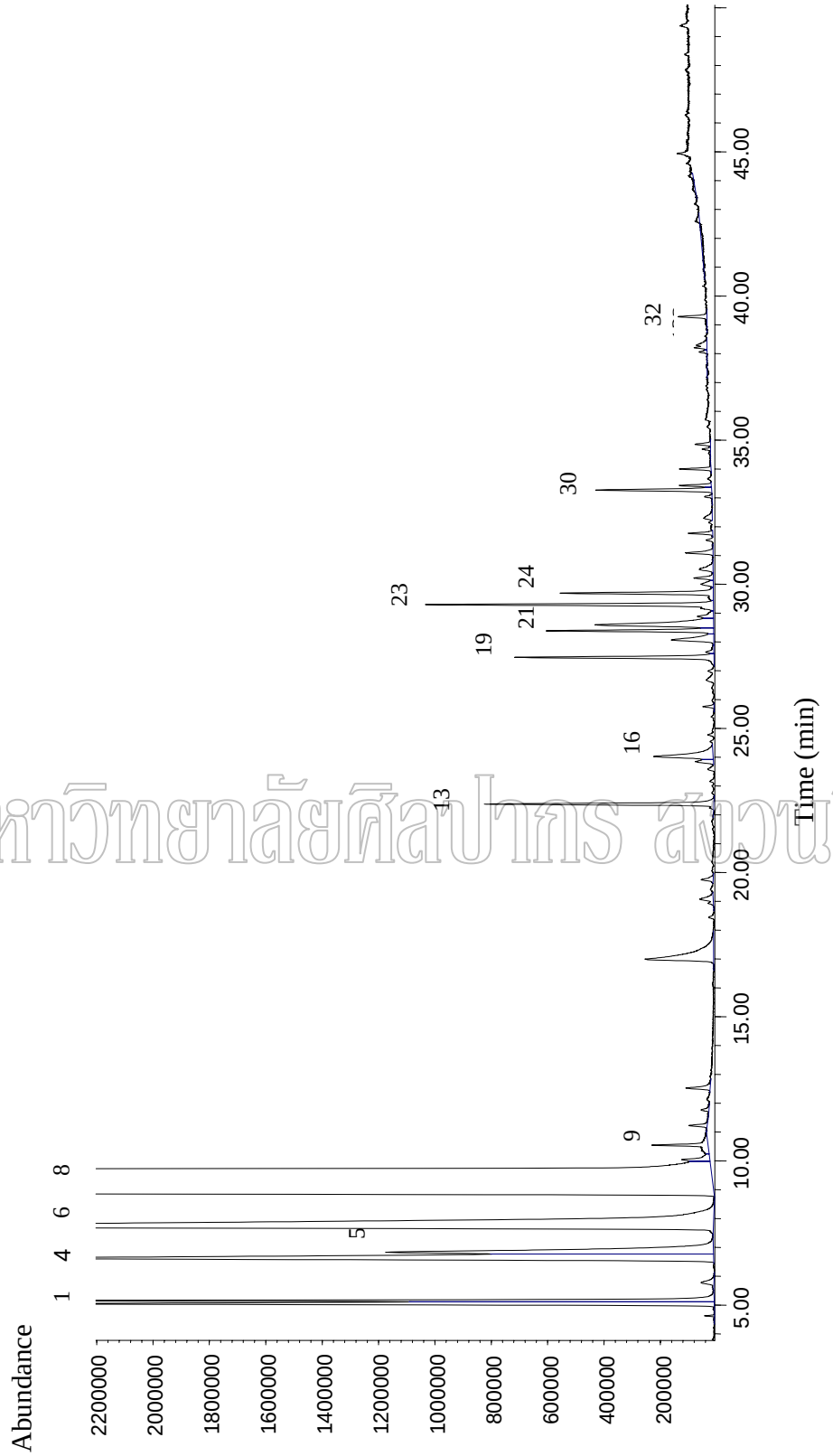


Figure 28 GC chromatogram of *C.maxima* peel essential oil from cold pressing method (CP) analysed by using carbowax column

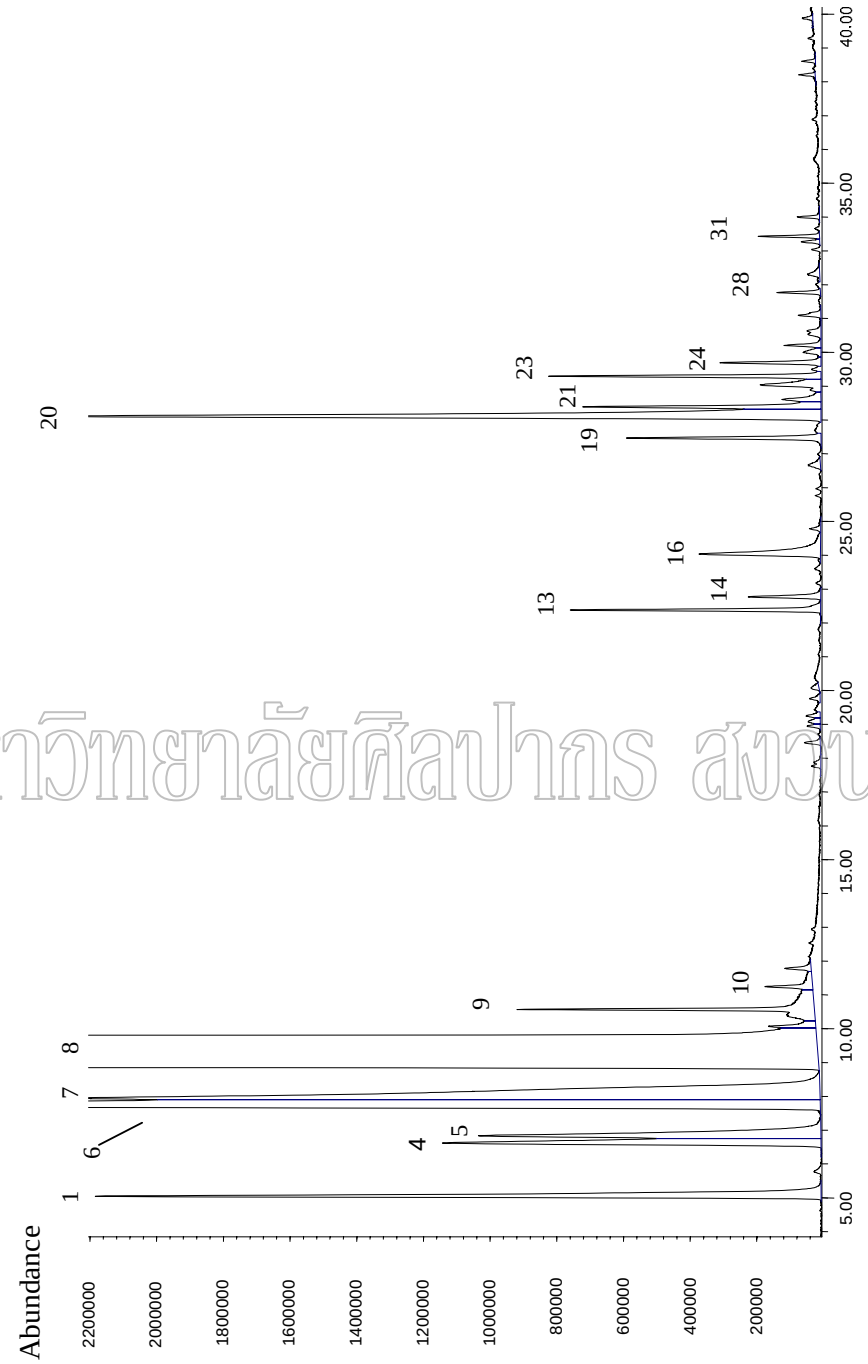


Figure 29 GC chromatogram of *C.maxima* peel essential oil from vacuum steam distillation (VP) analysed by using carbowax column

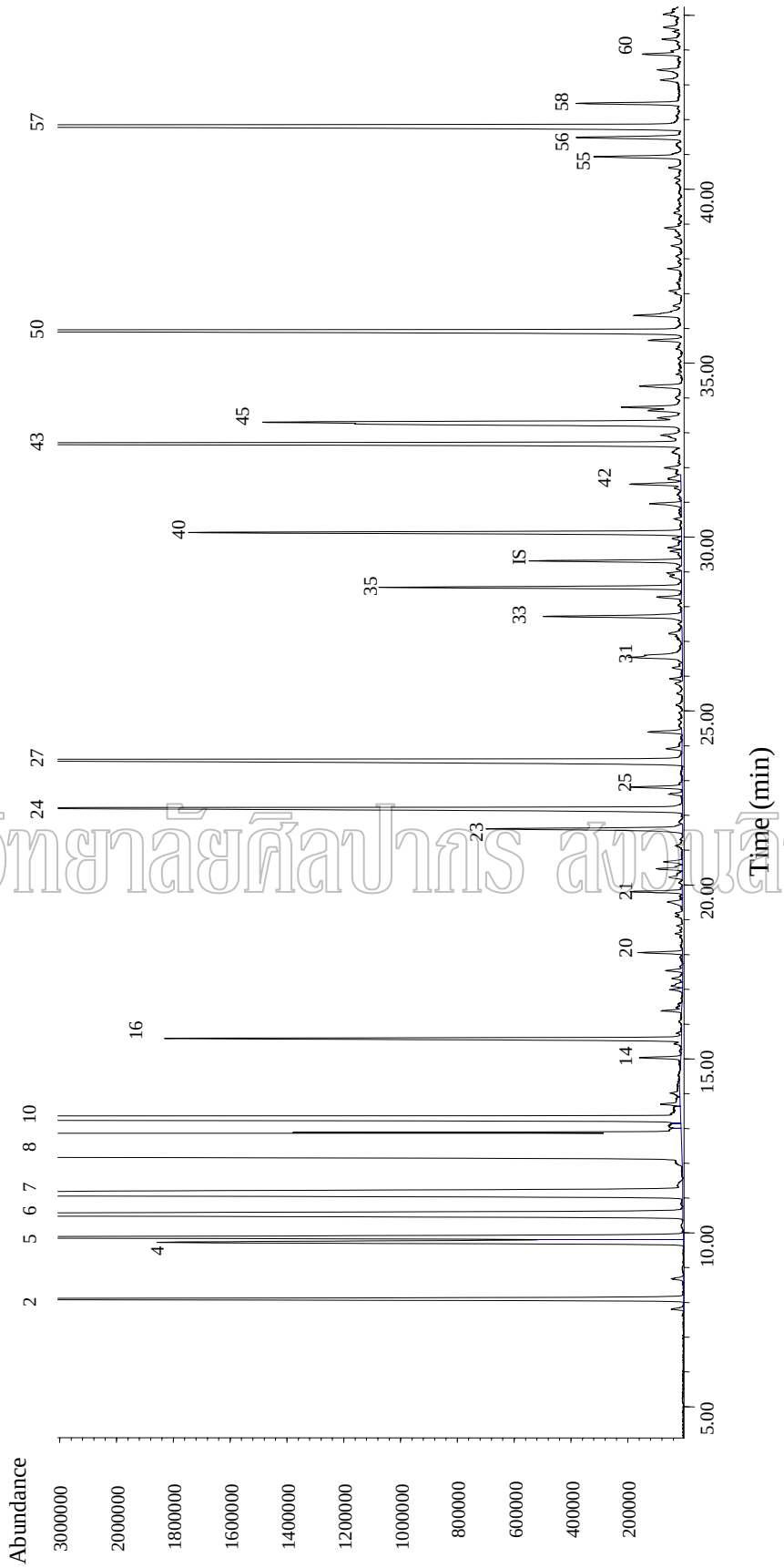


Figure 30 GC chromatogram of *C.maxima* flower essential oil from supercritical carbon dioxide extraction(SC-f) analysed by using DB-5 column, IS : internal standard (tetradecane)

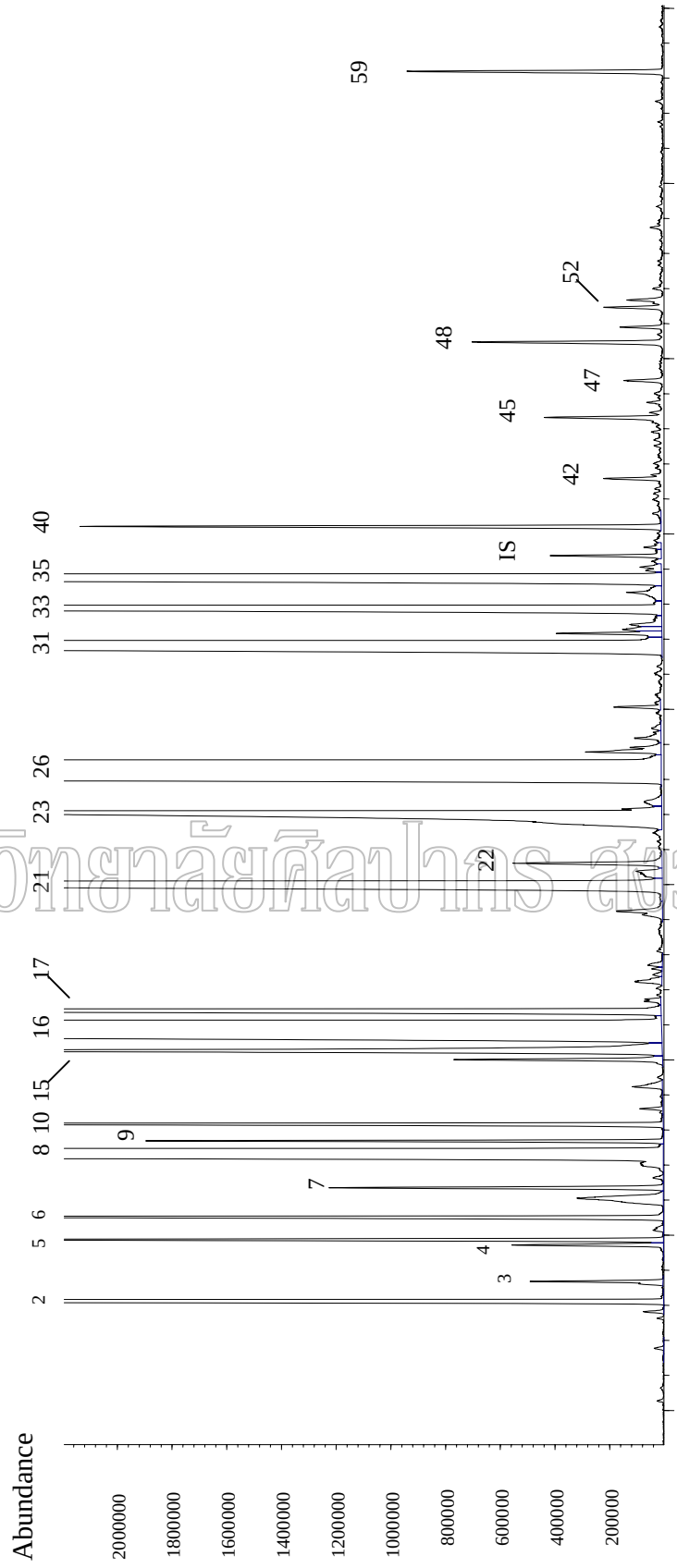


Figure 31 GC chromatogram of *C. aurantium* var. *amara* flower essential oil from solvent extraction (neroli) analysed by using DB-5 column, IS : internal standard (tetradecane)

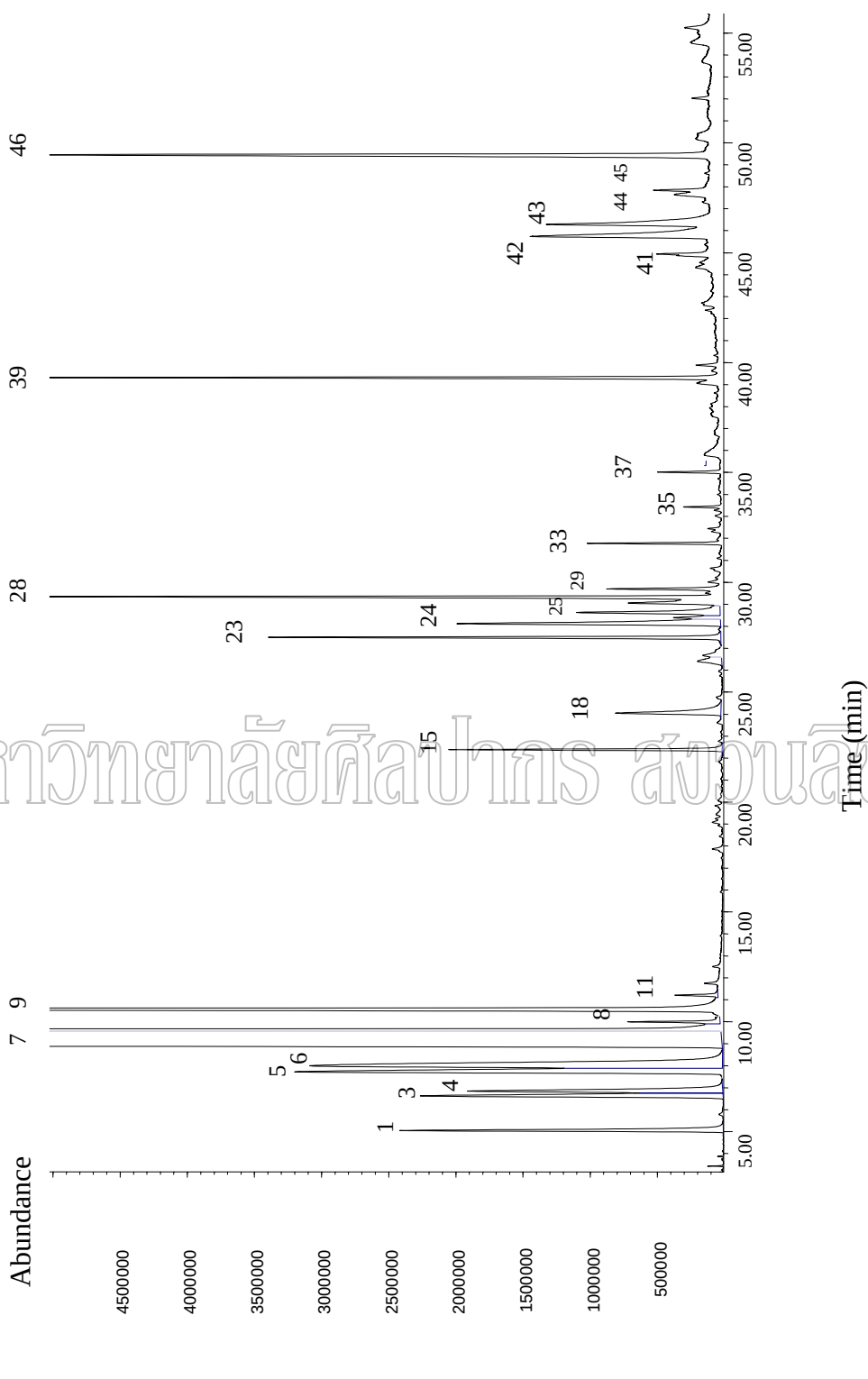


Figure 32 GC chromatogram of *C.maxima* flower essential oil from supercritical carbon dioxide extraction (SC-f) analysed by using carbowax column

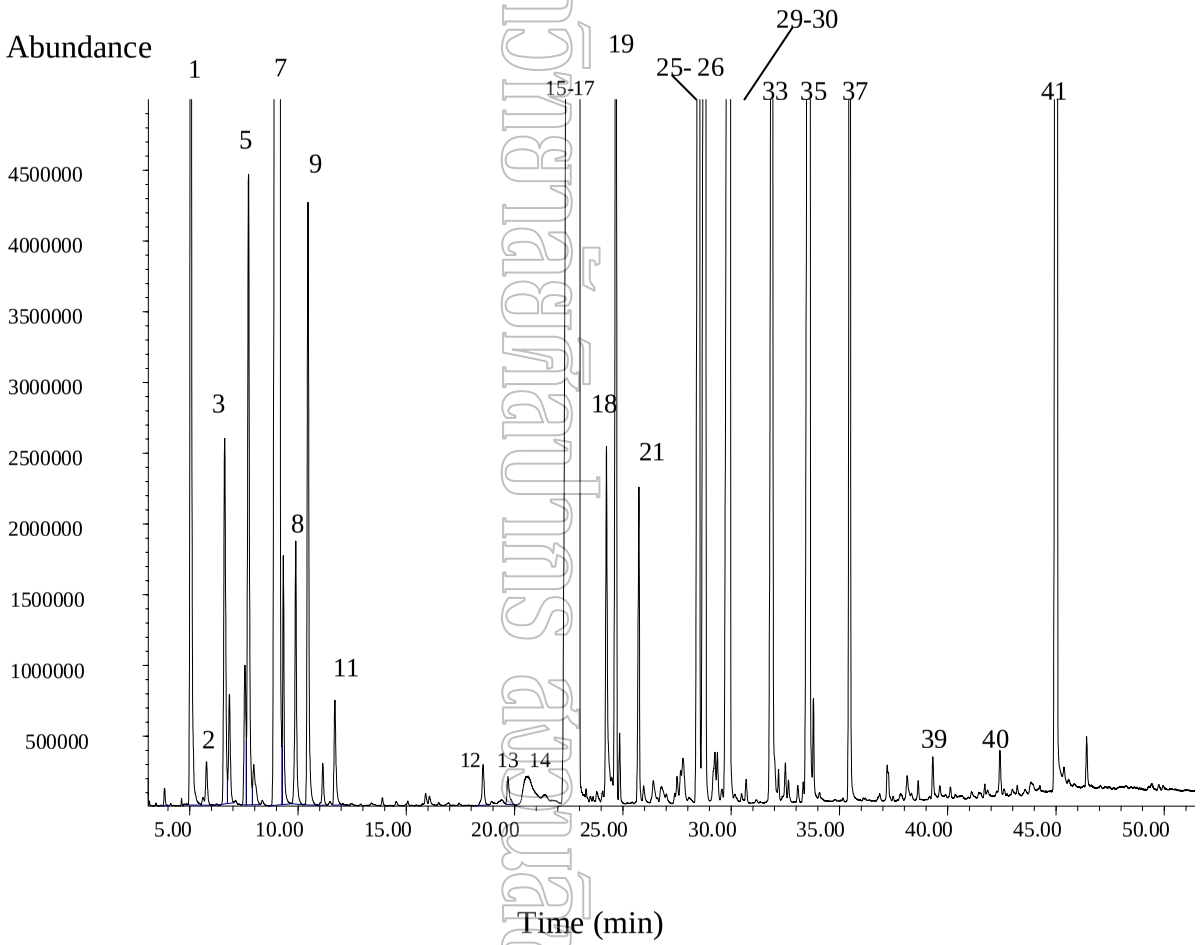
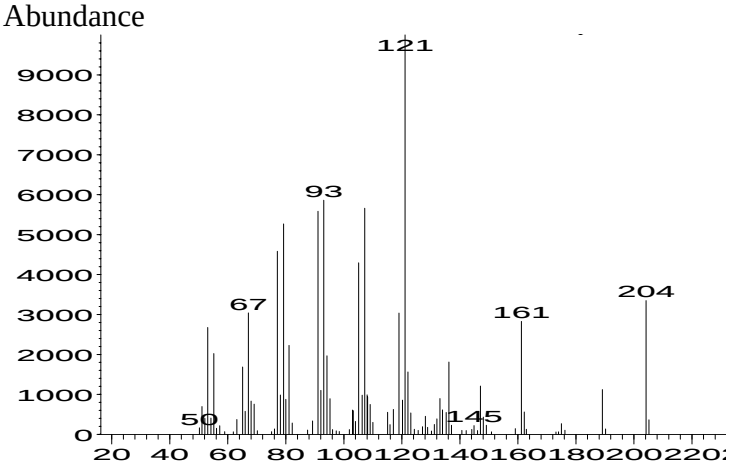


Figure 33 GC chromatogram of *C. aurantium* var. *amara* flower essential oil from solvent extraction (neroli) analysed by using carbowax column



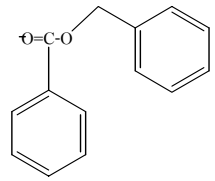
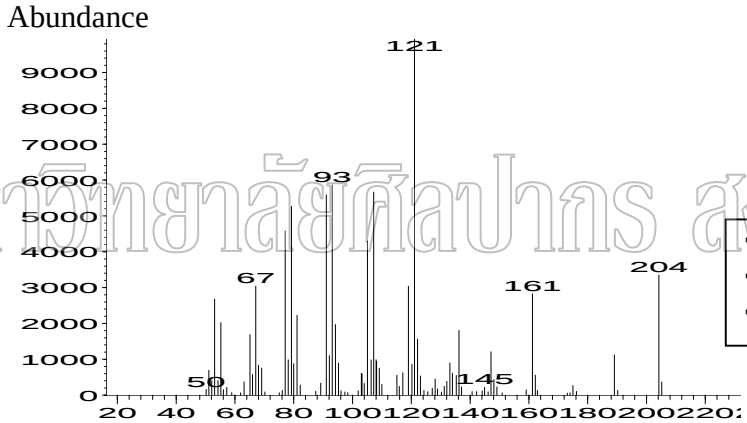


Table 12 Peak no. 59

Figure 34 Mass spectrum of identified benzyl benzoate



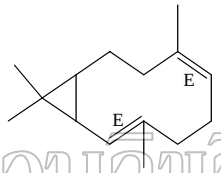
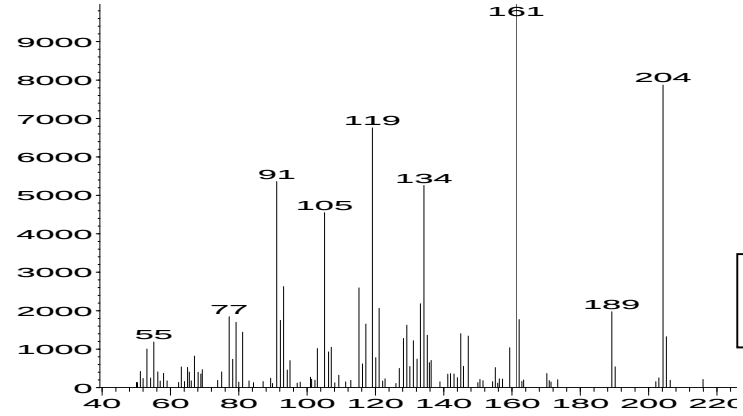


Table 8 Peak no. 41
Table 9 Peak no. 22
Table 13 Peak no. 27

Figure 35 Mass spectrum of identified bicyclogermacrene



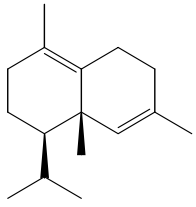
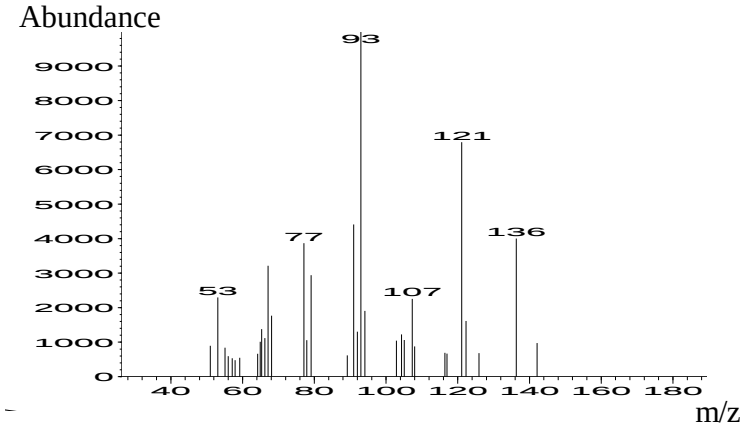


Table 8 Peak no. 43
Table 12 Peak no. 47

Figure 36 Mass spectrum of identified δ-(+)-cadinene



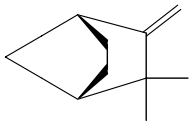
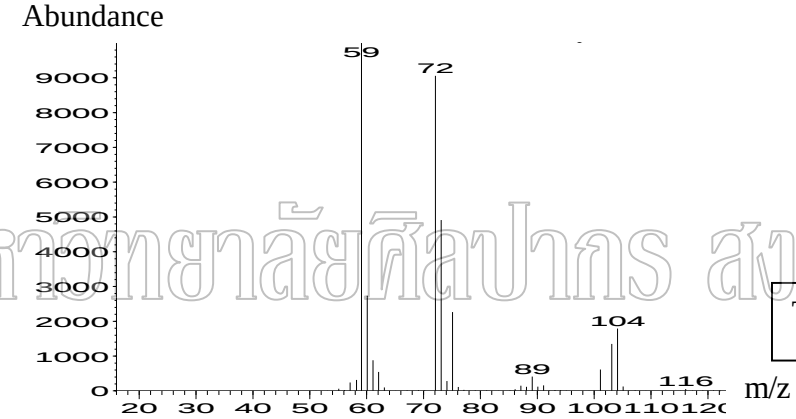


Table 8 Peak no. 3
Table 9 Peak no. 3
Table 12 Peak no. 3
Table 13 Peak no. 2

Figure 37 Mass spectrum of identified camphene



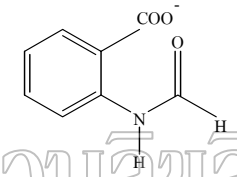
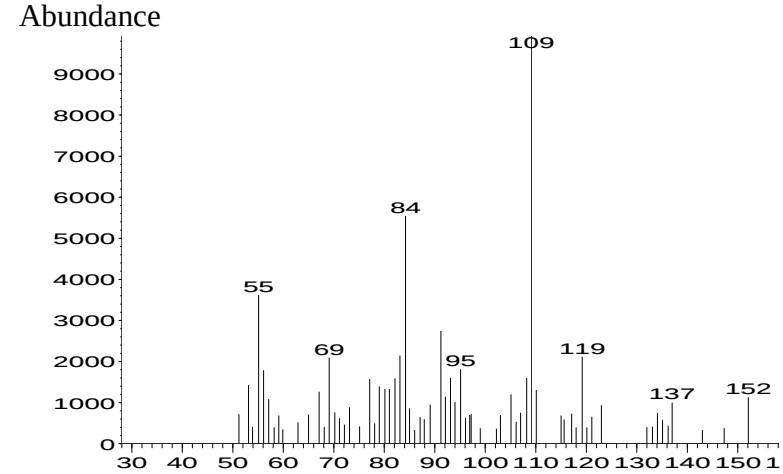


Table 13 Peak no. 21

Figure 38 Mass spectrum of identified carbitol



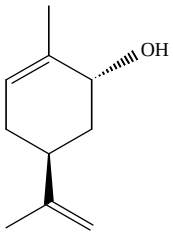


Table 9 Peak no. 30

Figure 39 Mass spectrum of identified (*E*)-(+)-carveol

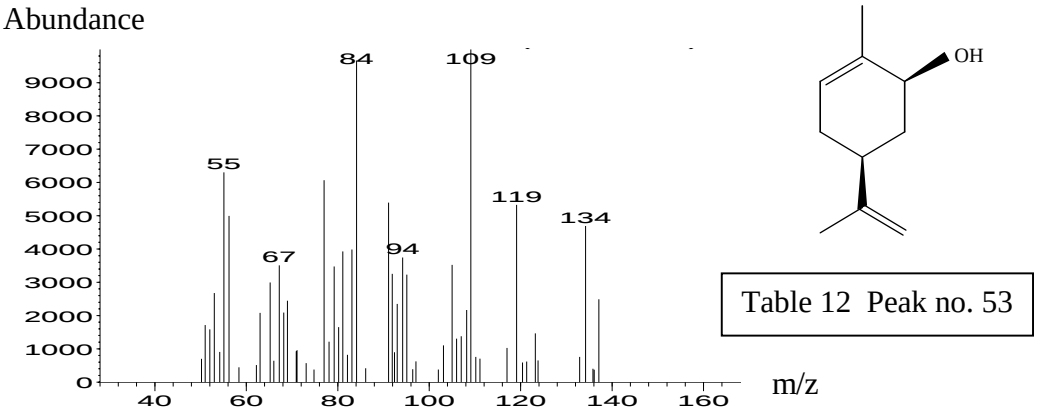


Figure 40 Mass spectrum of identified (*Z*)-(+)-carveol

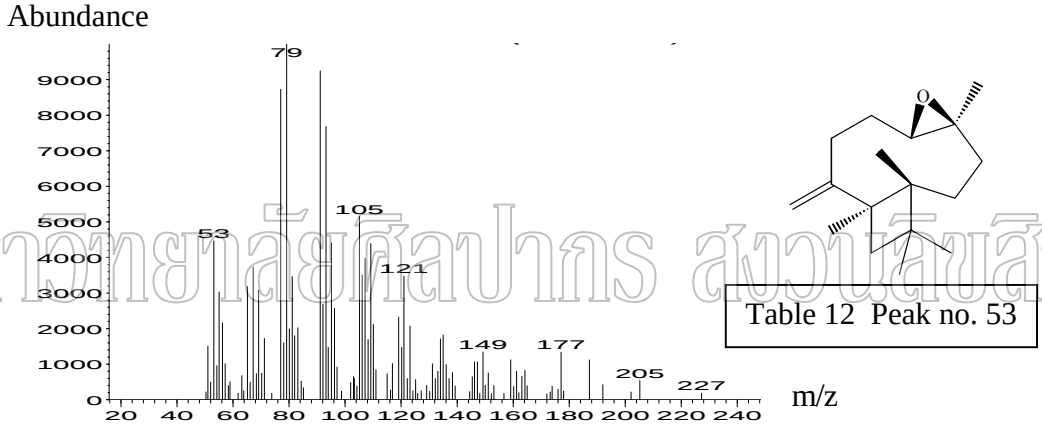


Figure 41 Mass spectrum of identified caryophyllene oxide

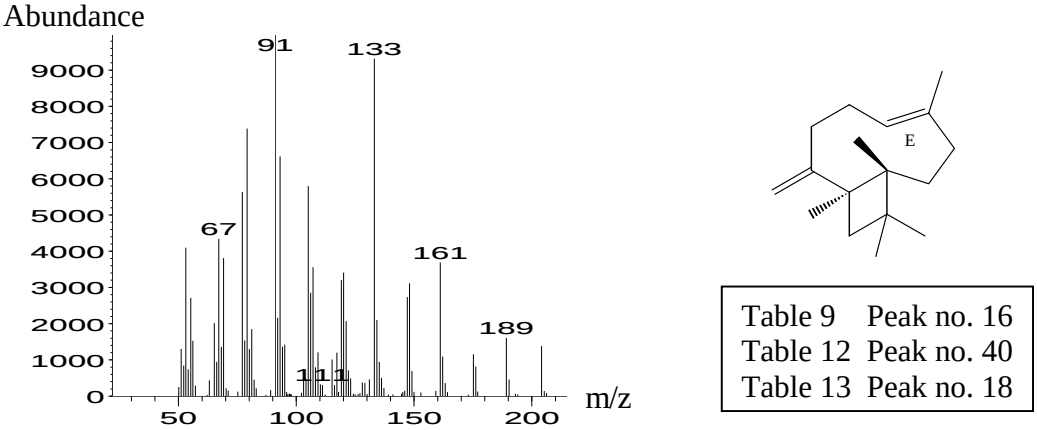


Figure 42 Mass spectrum of identified β-caryophyllene

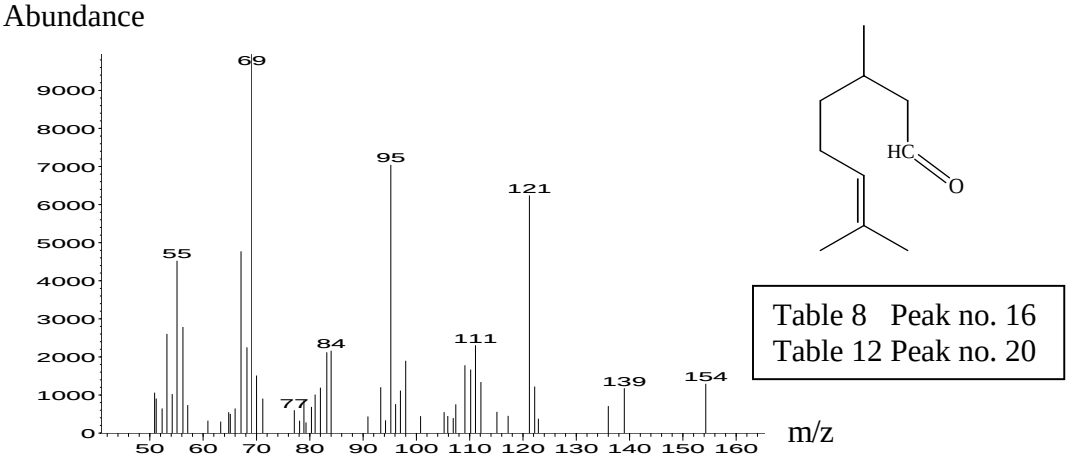


Table 8 Peak no. 16
Table 12 Peak no. 20

Figure 43 Mass spectrum of identified citronellal

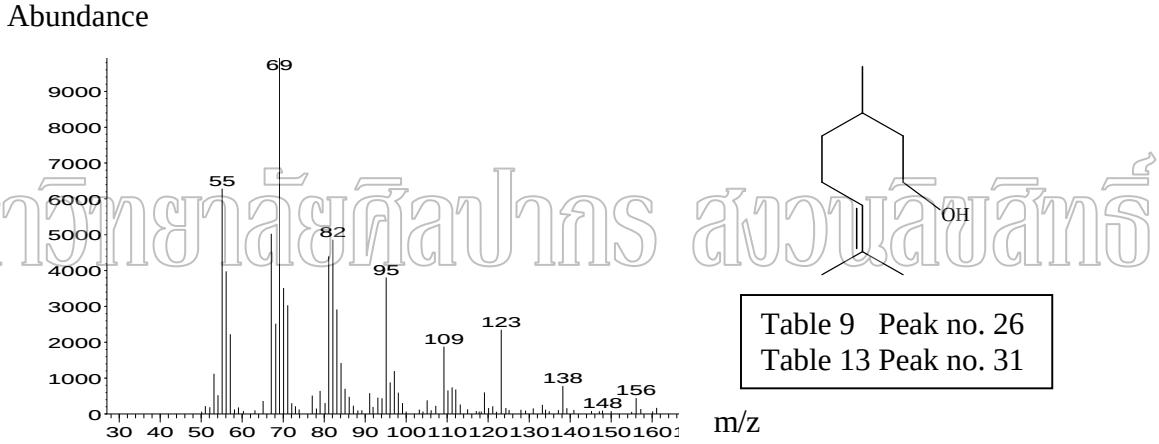


Table 9 Peak no. 26
Table 13 Peak no. 31

Figure 44 Mass spectrum of identified citronellol

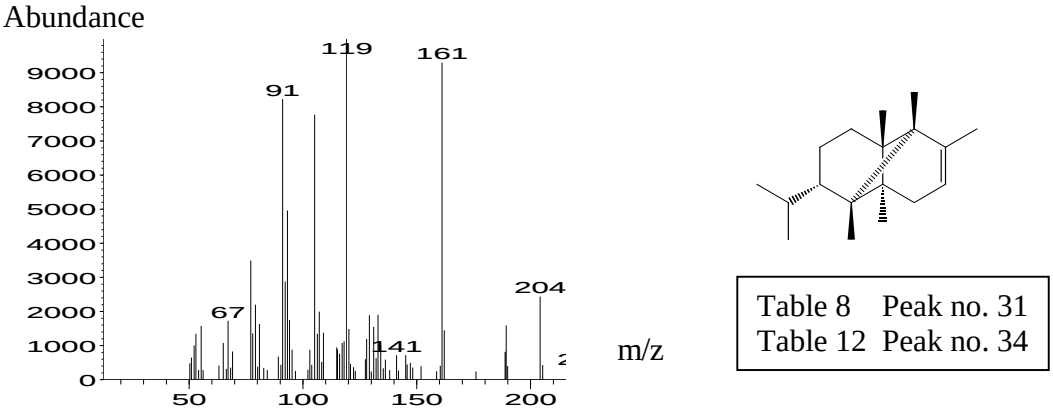
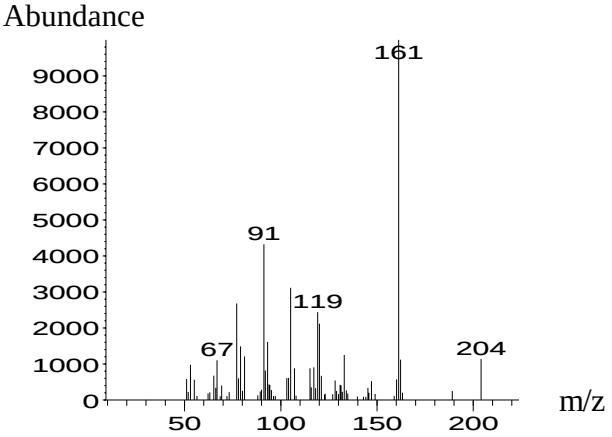


Table 8 Peak no. 31
Table 12 Peak no. 34

Figure 45 Mass spectrum of identified α –copaene



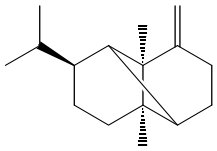
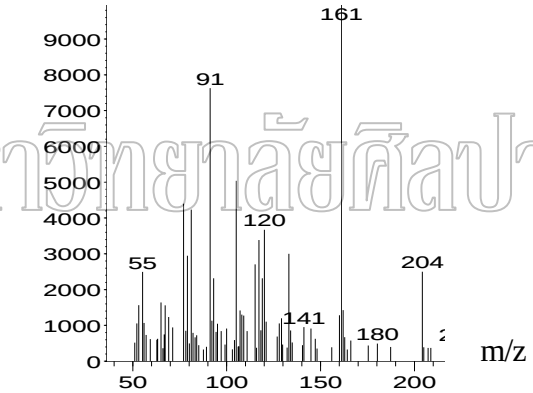


Table 8 Peak no. 37
Table12 Peak no. 41

Figure 46 Mass spectrum of identified β-copaene



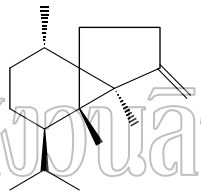
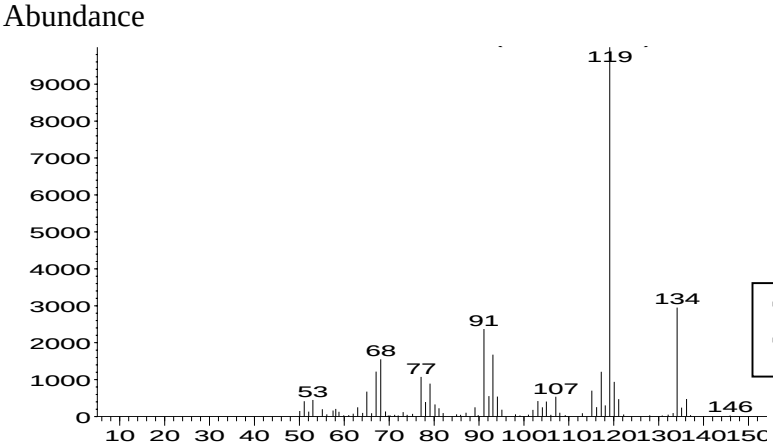


Table 8 Peak no. 33
Table12 Peak no. 36

Figure 47 Mass spectrum of identified β-(-)-cubebene



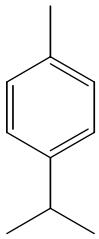
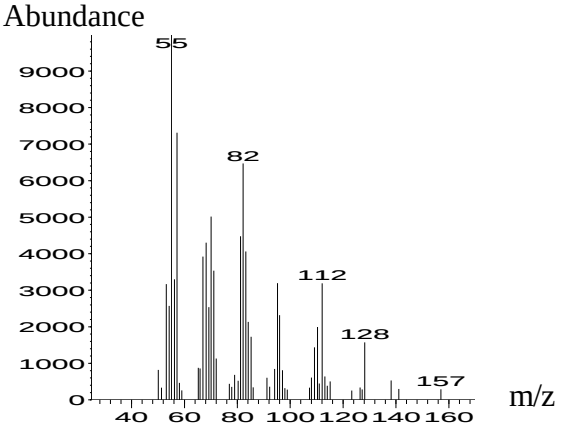


Table 9 Peak no. 10
Table 13 Peak no. 10

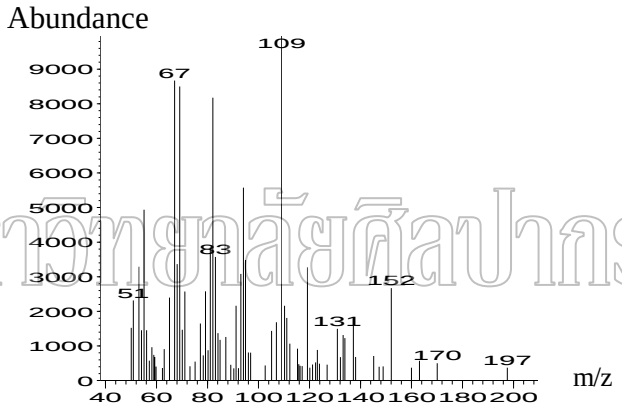
Figure 48 Mass spectrum of identified *p*-cymene



$CH_3(CH_2)_8CHO$

Table 8 Peak no. 19
Table12 Peak no. 22
Table13 Peak no. 14

Figure 49 Mass spectrum of identified *n*-decanal



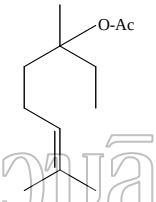
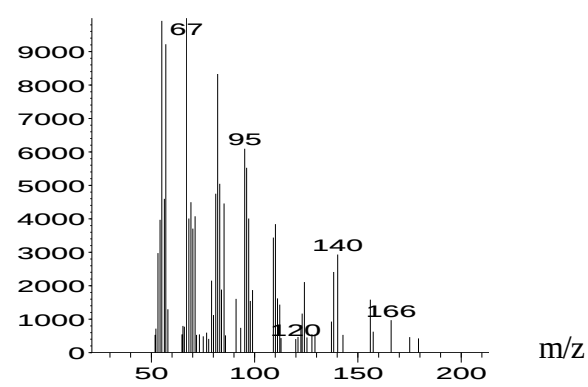


Table 12 Peak no. 28

Figure 50 Mass spectrum of identified dihydrolinalyl acetate



$CH_3(CH_2)_{10}C(=O)H$

Table 8 Peak no. 34
Table12 Peak no. 38

Figure 51 Mass spectrum of identified dodecanal

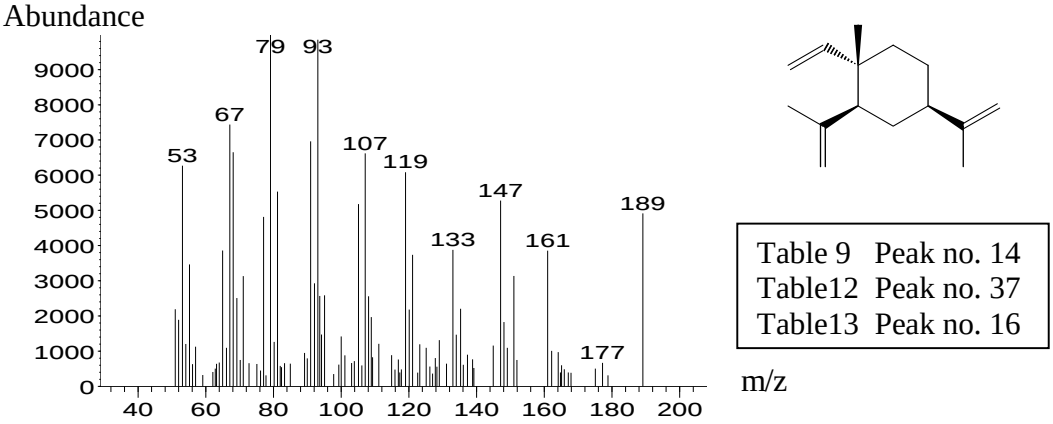


Figure 52 Mass spectrum of identified β-(-)-elemene

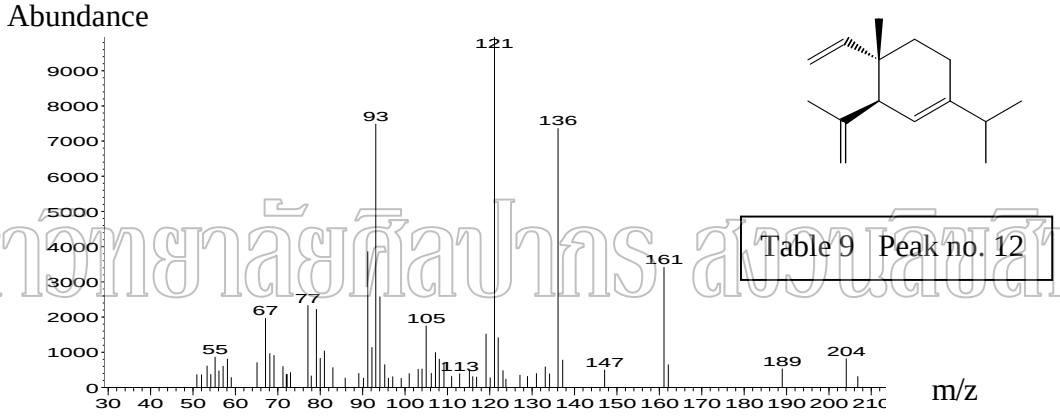


Figure 53 Mass spectrum of identified δ-elemene

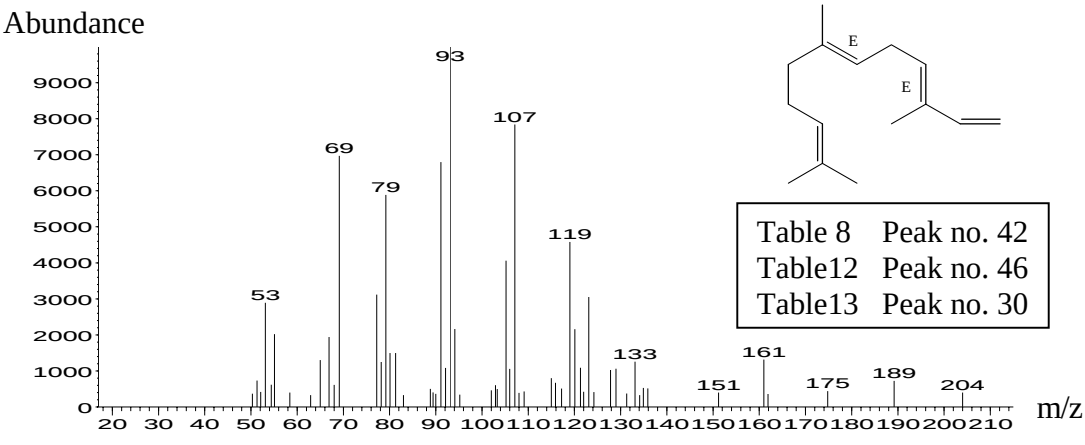


Figure 54 Mass spectrum of identified (*E*,*E*)-α –farnesene

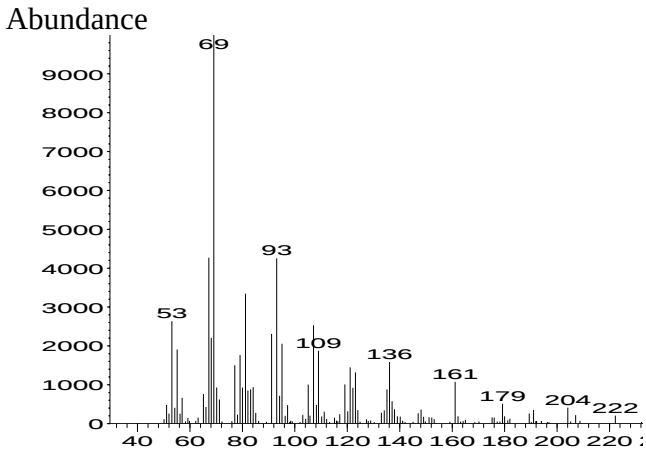
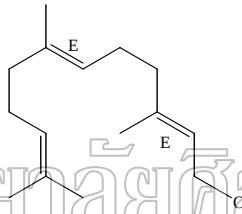
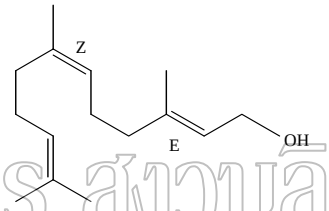


Figure 55 Mass spectrum of identified farnesol



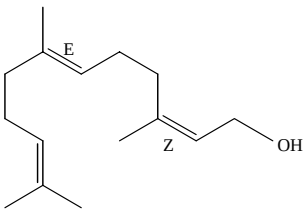
(*E*,*E*)-farnesol

Table 8	Peak no.	50
Table 9	Peak no.	37
Table12	Peak no.	57
Table13	Peak no.	45



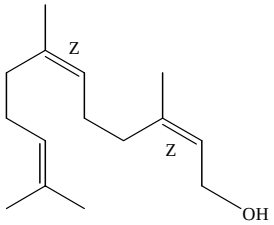
(*E*,*Z*)-farnesol

Table 8	Peak no.	5
Table12	Peak no.	58
Table13	Peak no.	46



(*Z*,*E*)-farnesol

Table 8	Peak no.	48
Table 9	Peak no.	37
Table12	Peak no.	55
Table13	Peak no.	43



(*Z*,*Z*)-farnesol

Table 8	Peak no.	49
Table12	Peak no.	56
Table13	Peak no.	44

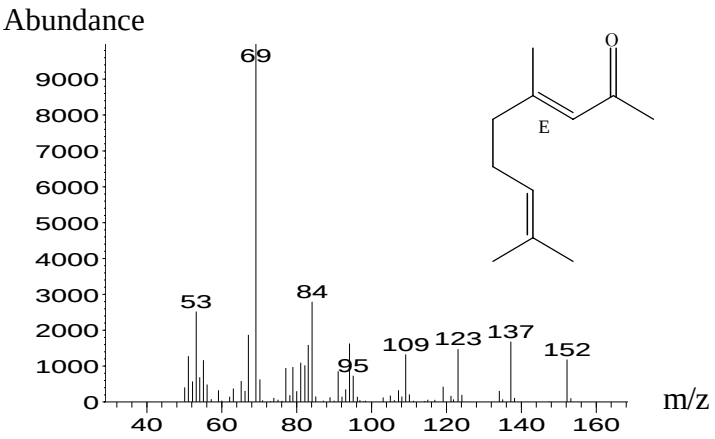


Table 8	Peak no. 25
Table 9	Peak no. 23
Table12	Peak no. 27
Table13	Peak no. 28

Figure 56 Mass spectrum of identified geranial

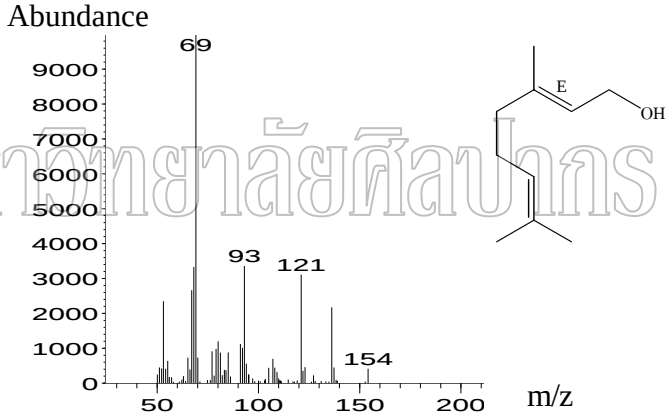


Table 8	Peak no. 24
Table 9	Peak no. 31
Table12	Peak no. 25
Table13	Peak no. 35

Figure 57 Mass spectrum of identified geraniol

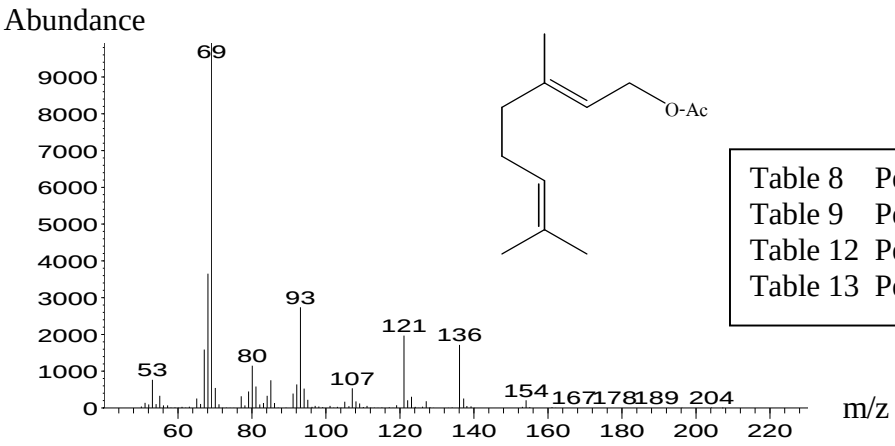


Table 8	Peak no. 32
Table 9	Peak no. 24
Table 12	Peak no. 35
Table 13	Peak no. 29

Figure 58 Mass spectrum of identified geranyl acetate

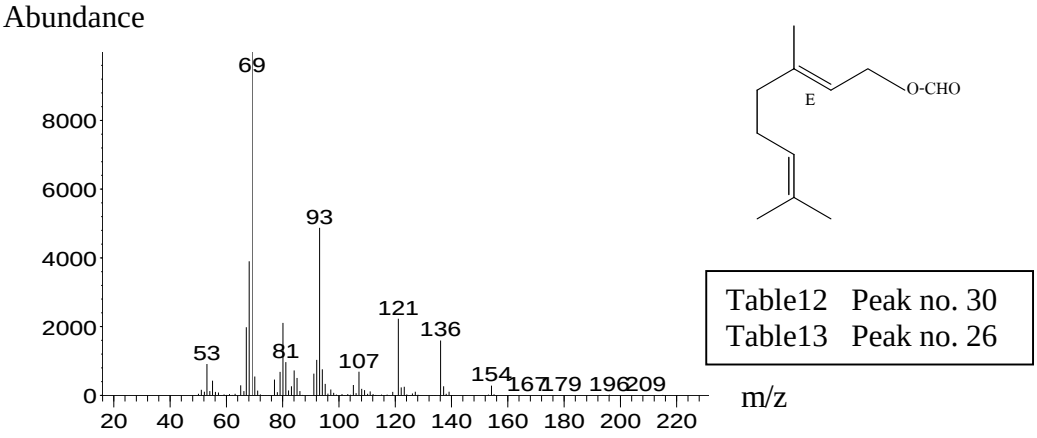


Figure 59 Mass spectrum of identified geranyl formate

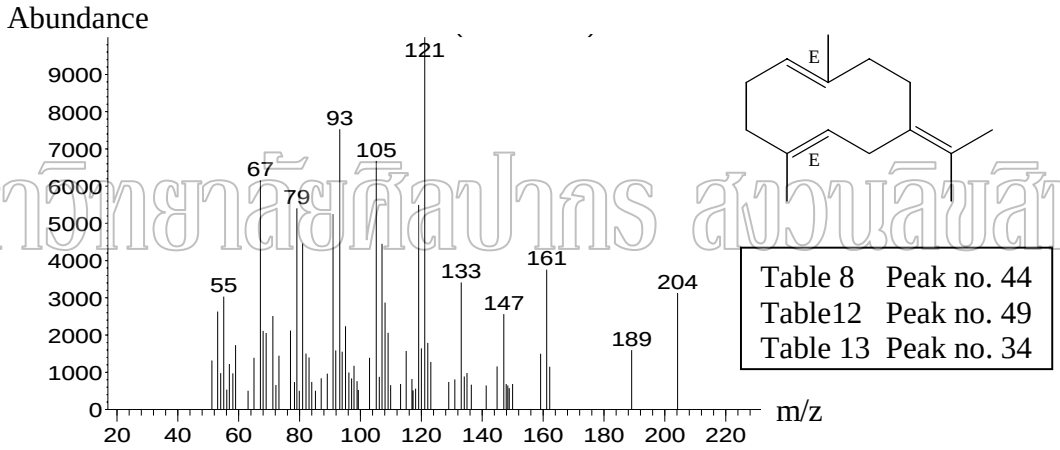


Figure 60 Mass spectrum of identified germacrene B

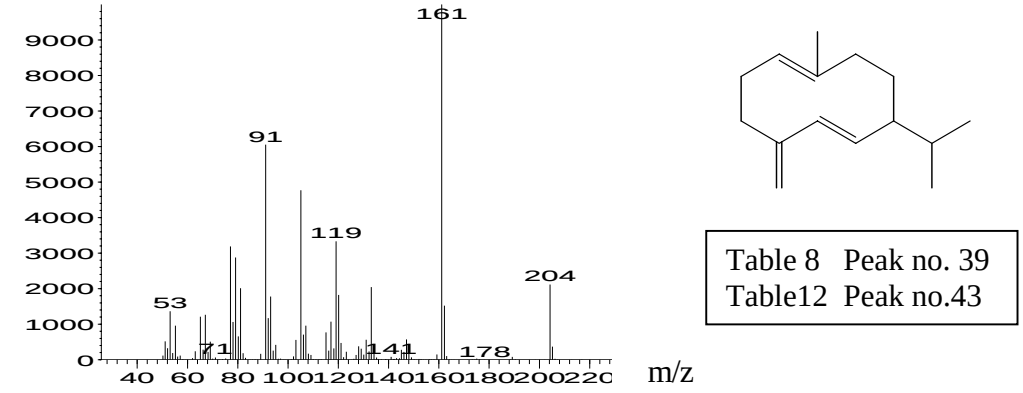


Figure 61 Mass spectrum of identified germacrene D

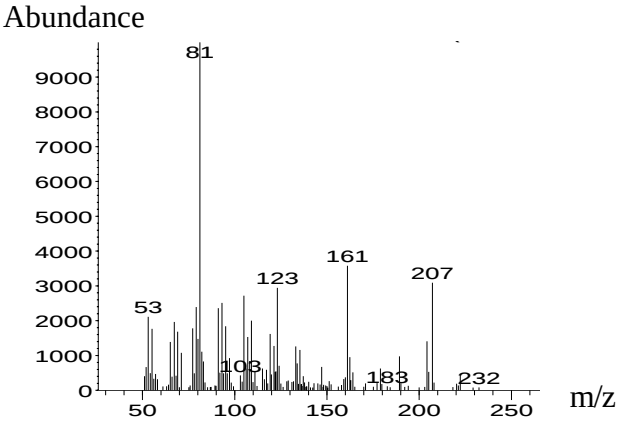


Table 8	Peak no.46
Table12	Peak no. 51

Figure 62 Mass spectrum of identified germacrene D-4-ol

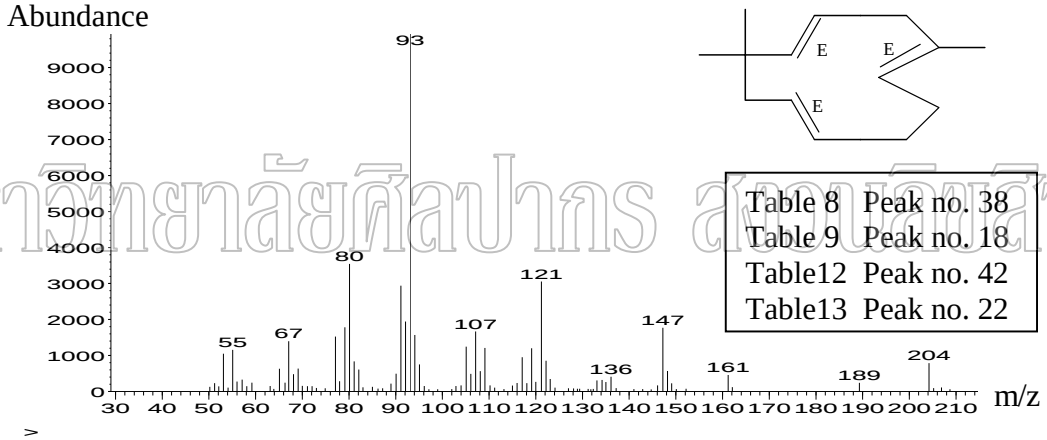


Table 8	Peak no. 38
Table 9	Peak no. 18
Table12	Peak no. 42
Table13	Peak no. 22

Figure 63 Mass spectrum of identified α –humulene

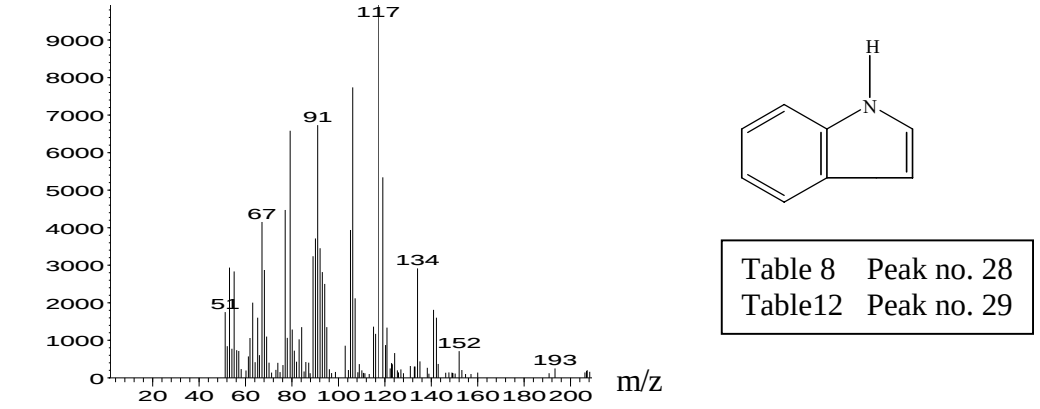
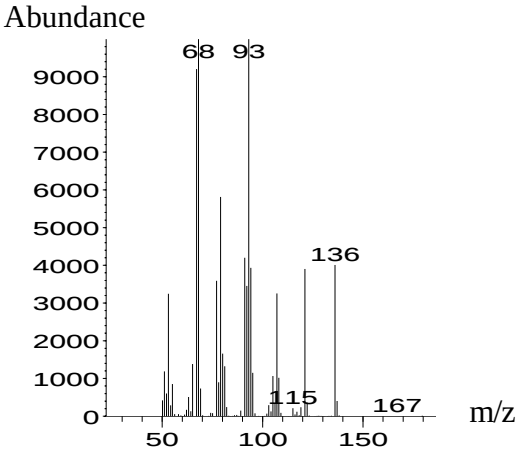


Table 8	Peak no. 28
Table12	Peak no. 29

Figure 64 Mass spectrum of identified indole



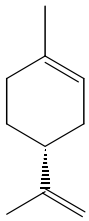
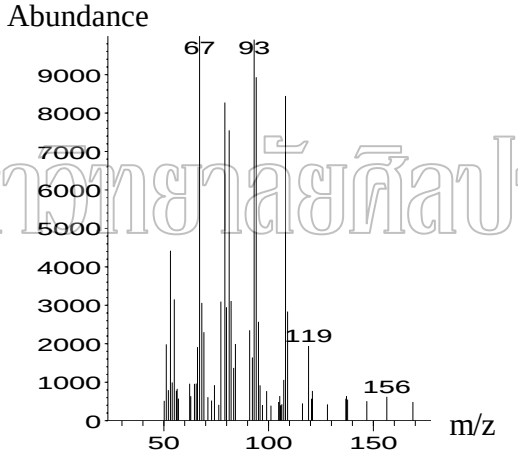


Table 8	Peak no. 8
Table 9	Peak no. 8
Table12	Peak no. 8
Table13	Peak no. 7

Figure 65 Mass spectrum of identified *R*-(+)-limonene



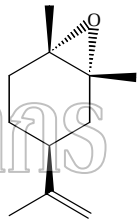
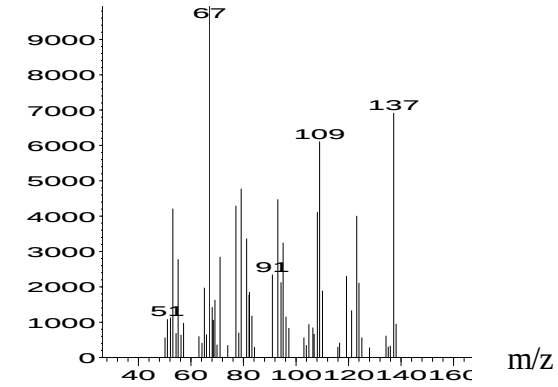


Table 8	Peak no. 15
Table12	Peak no. 19

Figure 66 Mass spectrum of identified (*E*)-(+)-limonene oxide



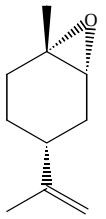


Table 8	Peak no. 14
Table12	Peak no. 18

Figure 67 Mass spectrum of identified (*Z*)-(-)-limonene oxide

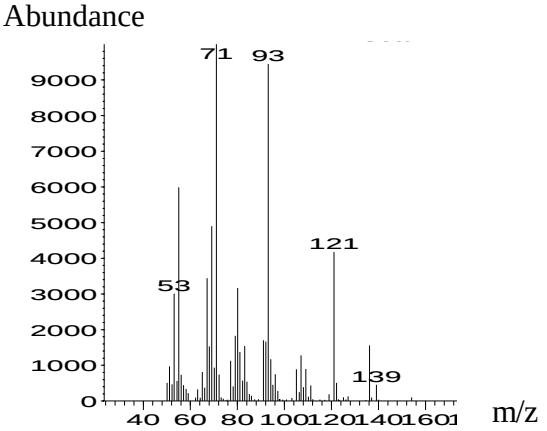


Table 8	Peak no.13
Table 9	Peak no.13
Table12	Peak no.16
Table13	Peak no.15

Figure 68 Mass spectrum of identified linalool

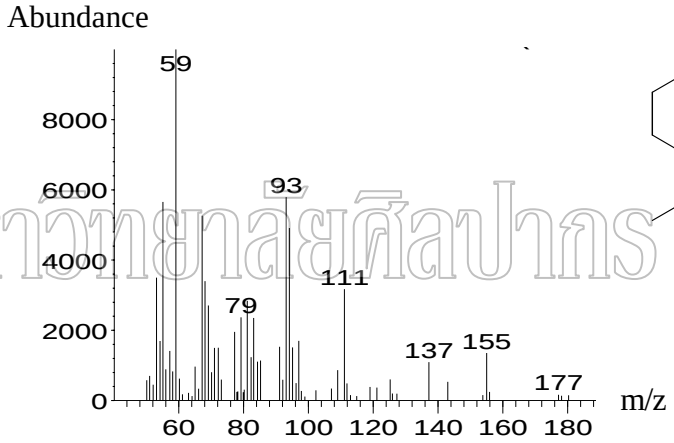


Table 13	Peak no.13

Figure 69 Mass spectrum of identified (*Z*)-(-)-linalool oxide

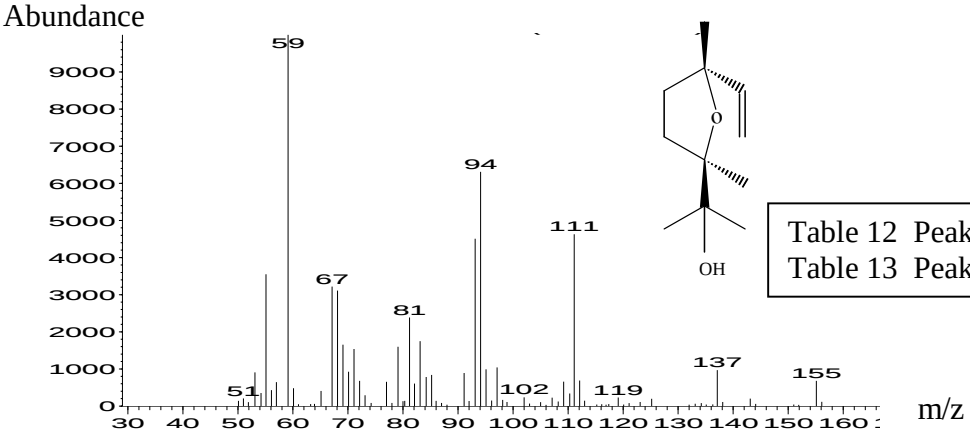


Table 12	Peak no. 13
Table 13	Peak no. 12

Figure 70 Mass spectrum of identified (*E*)-(+)-linalool oxide

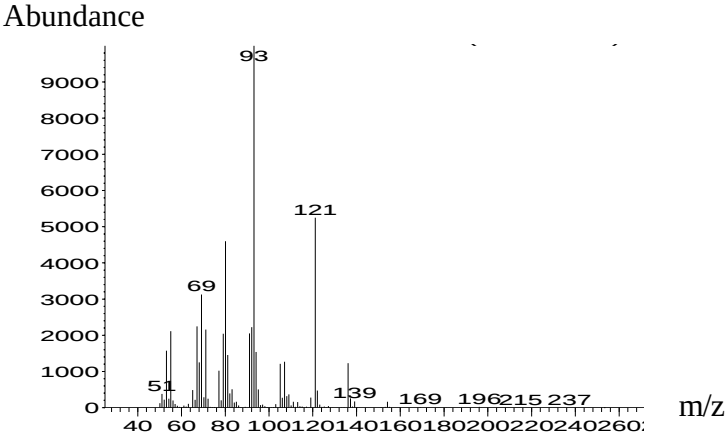


Table 9	Peak no. 15
Table12	Peak no. 26
Table13	Peak no. 17

Figure 71 Mass spectrum of identified linalyl acetate

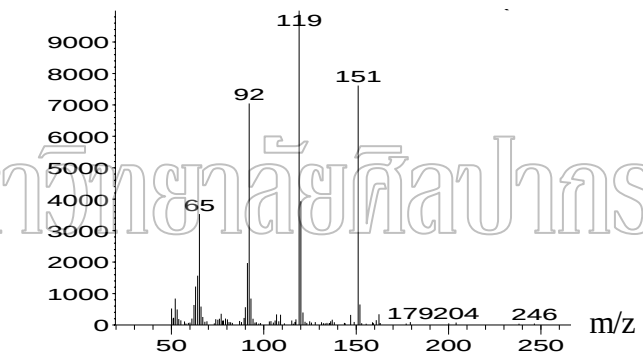


Table 8	Peak no. 28
Table12	Peak no. 31

Figure 72 Mass spectrum of identified methyl antranilate

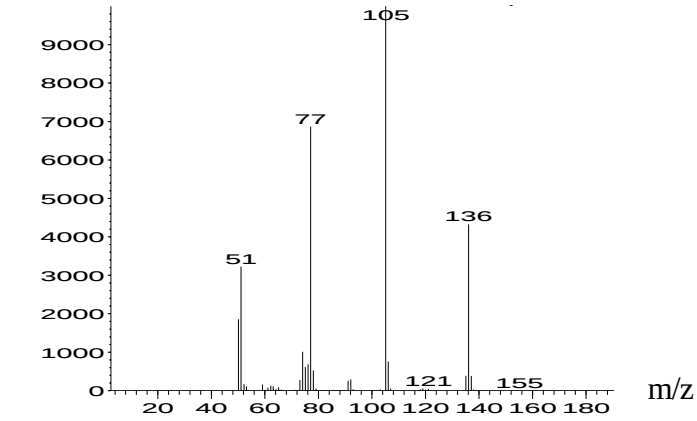


Table12	Peak no.15
Table13	Peak no.19

Figure 73 Mass spectrum of identified methyl benzoate

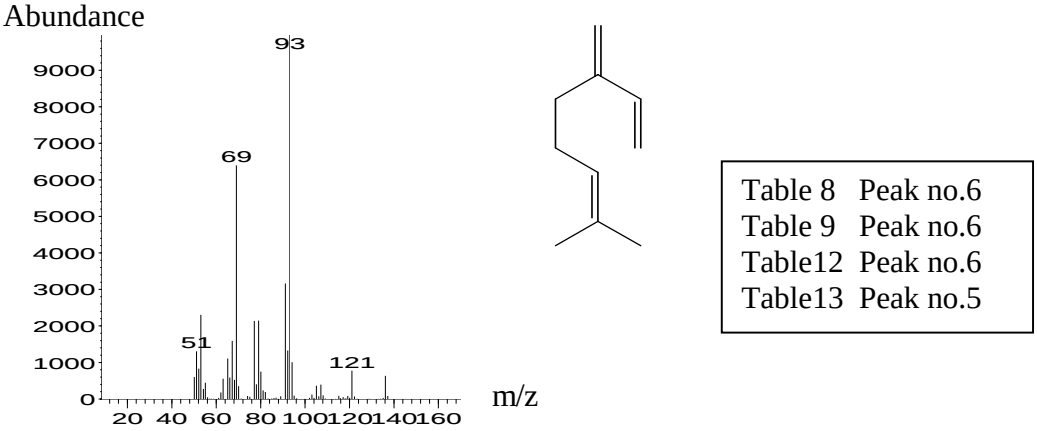


Figure 74 Mass spectrum of identified myrcene

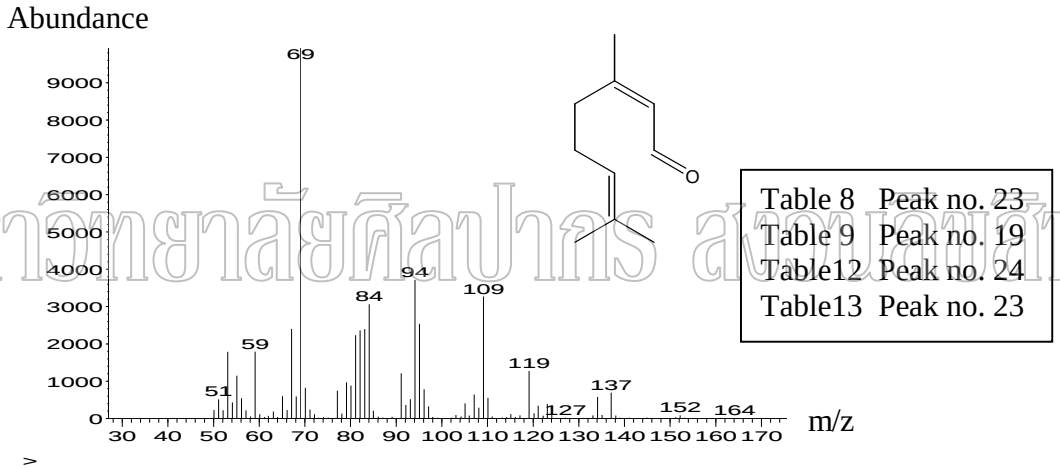


Figure 75 Mass spectrum of identified neral

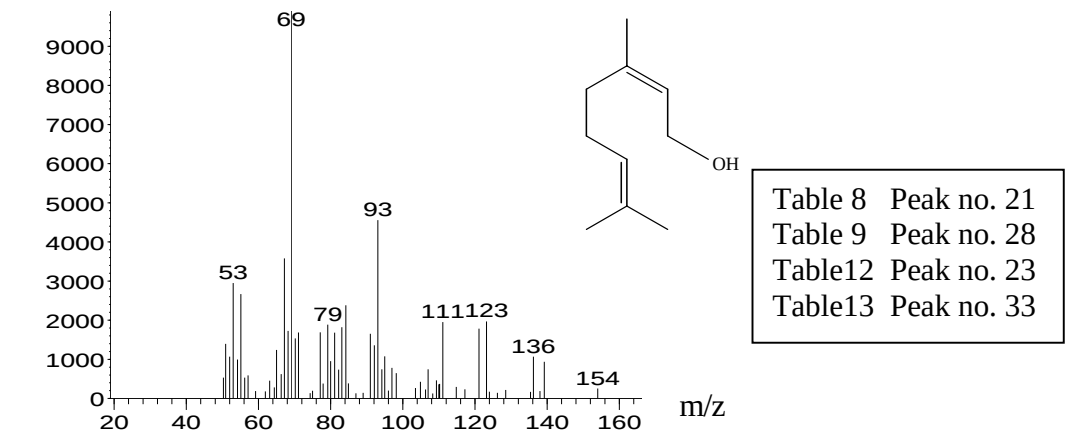


Figure 76 Mass spectrum of identified nerol

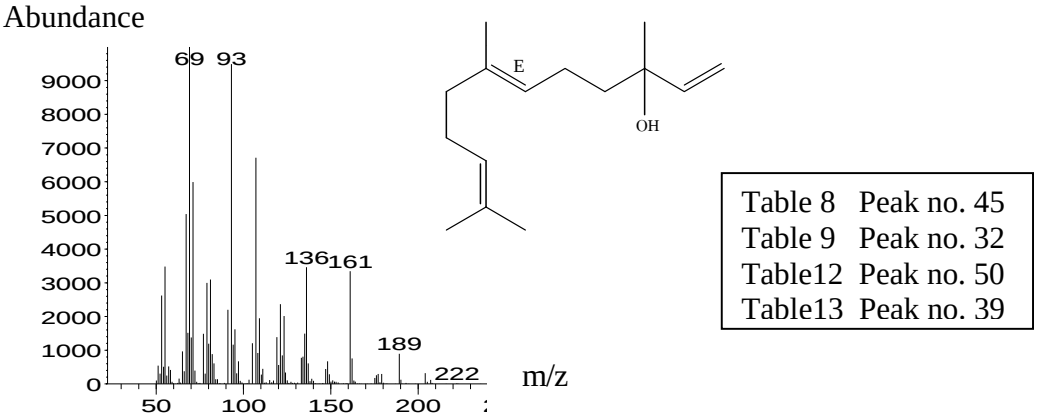


Figure 77 Mass spectrum of identified (*E*)-nerolidol

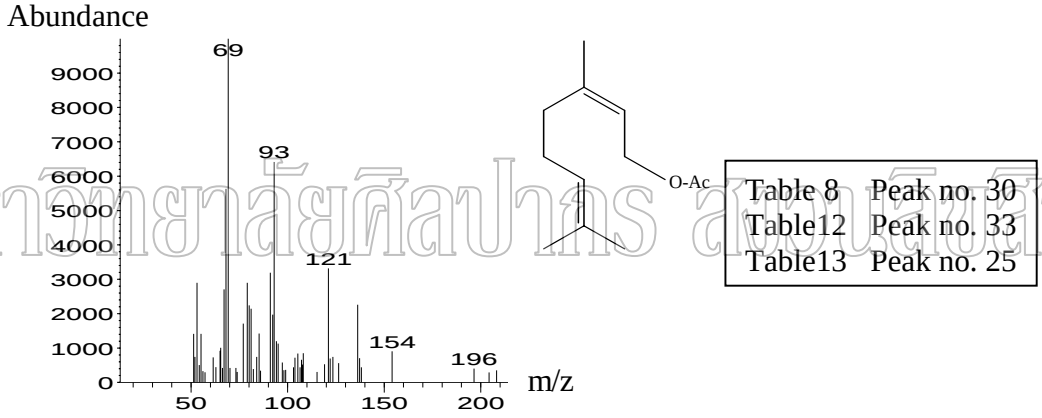


Figure 78 Mass spectrum of identified neryl acetate

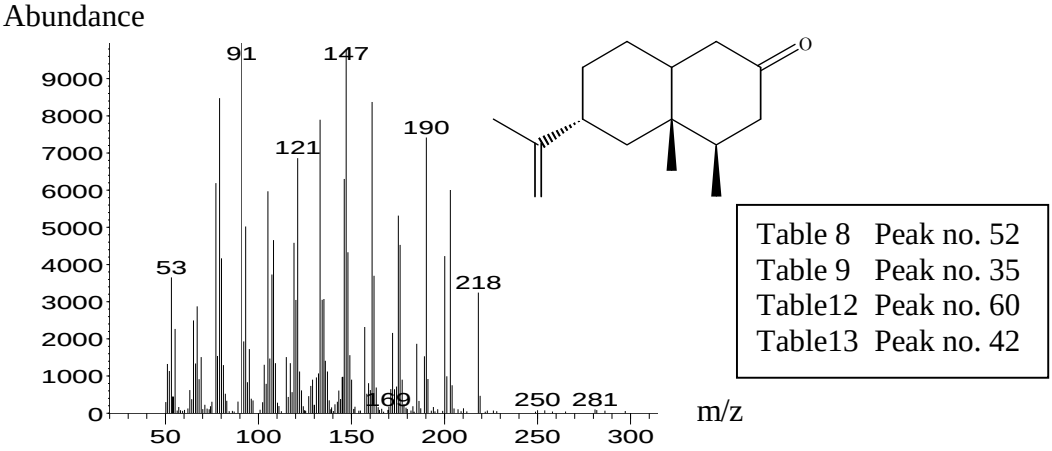
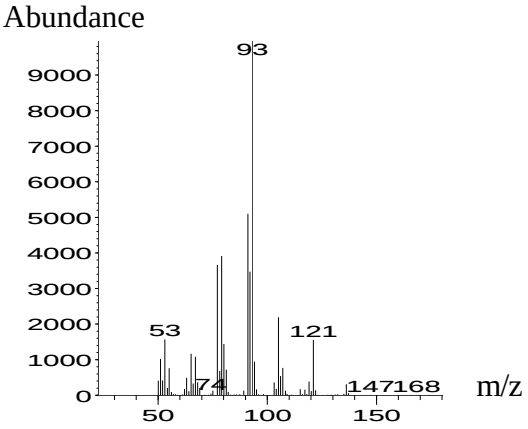


Figure 79 Mass spectrum of identified (+)-nootkatone



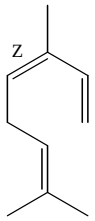
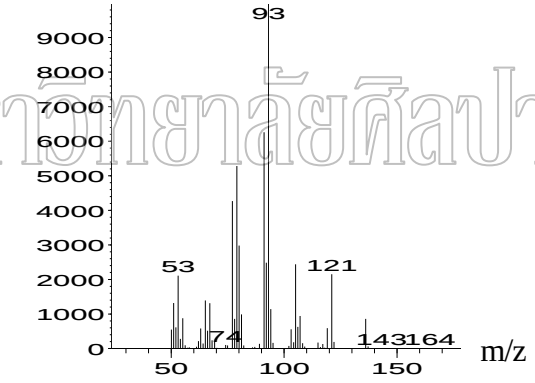


Table12	Peak no.9
Table13	Peak no.8

Figure 80 Mass spectrum of identified (*Z*)-β-ocimene



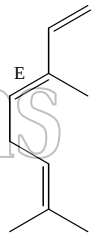
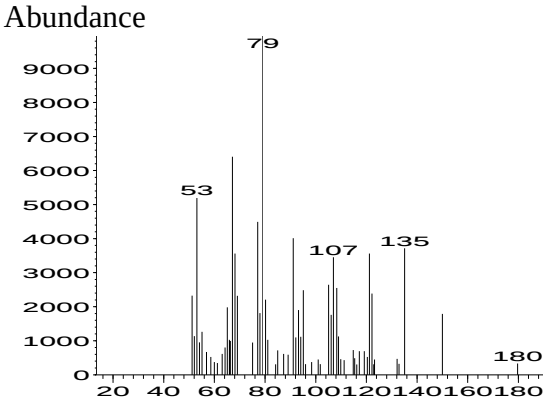


Table 8	Peak no. 9
Table 9	Peak no. 9
Table12	Peak no.10
Table13	Peak no. 9

Figure 81 Mass spectrum of identified (*E*)-β-ocimene



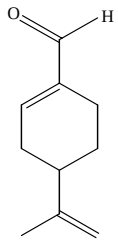


Table 8	Peak no. 26
Table 9	Peak no. 27
Table13	Peak no. 32

Figure 82 Mass spectrum of identified perilla aldehyde

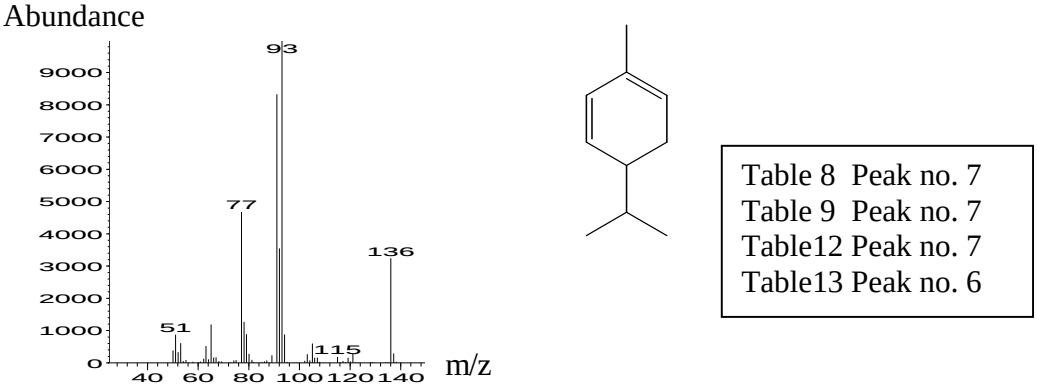


Figure 83 Mass spectrum of identified α –phellandrene

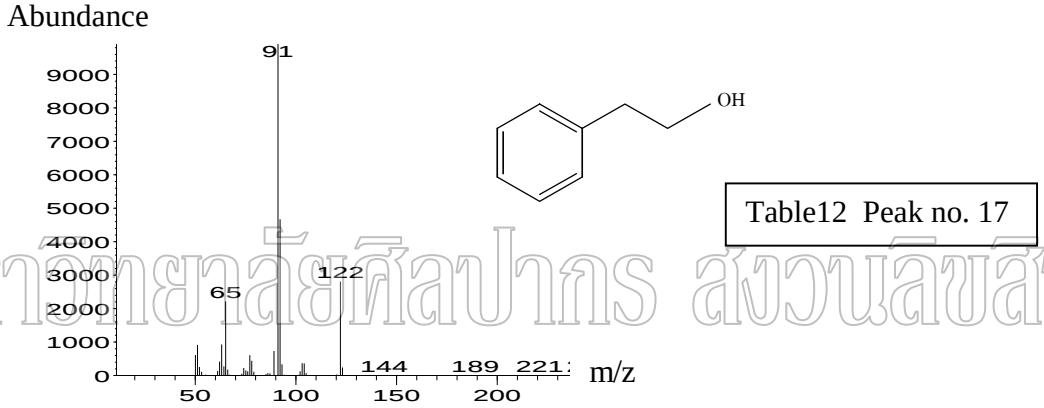


Figure 84 Mass spectrum of identified phenyl ethyl alcohol

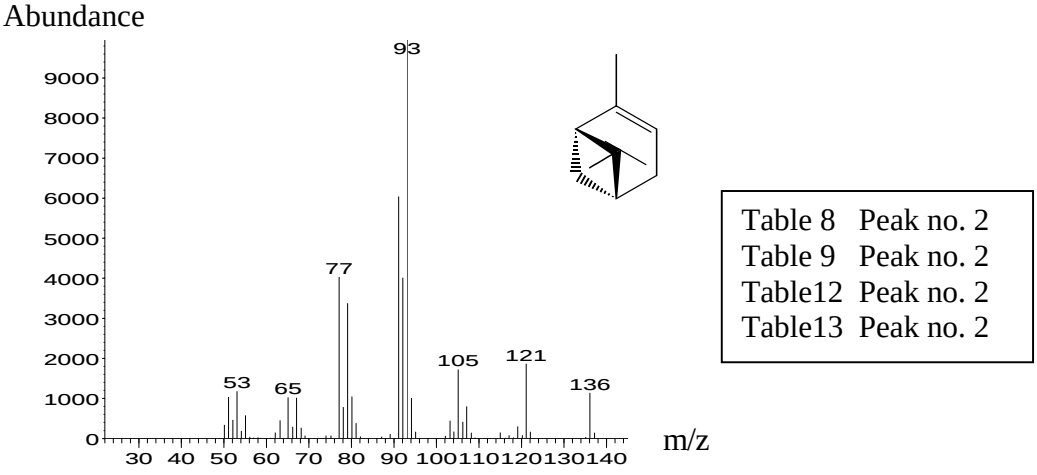
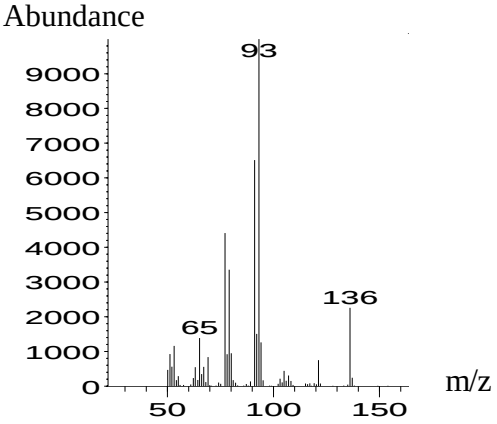


Figure 85 Mass spectrum of identified α –(-)-pinene



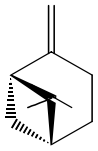
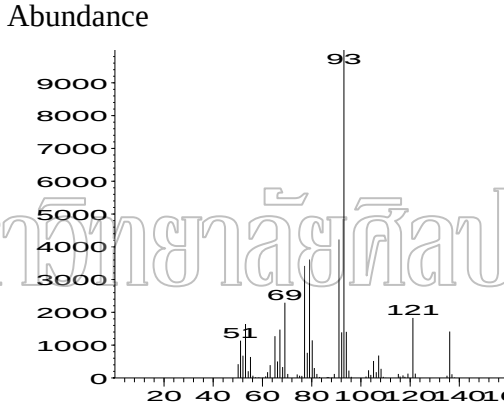


Table 8	Peak no. 5
Table 9	Peak no. 4
Table12	Peak no. 5
Table13	Peak no. 3

Figure 86 Mass spectrum of identified β-(+)-pinene



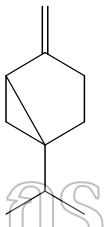
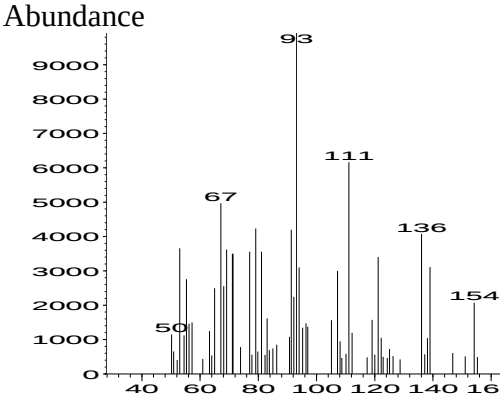


Table 8	Peak no. 4
Table 9	Peak no. 5
Table12	Peak no. 4
Table13	Peak no. 4

Figure 87 Mass spectrum of identified sabinene



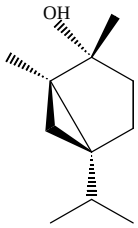


Table 8	Peak no. 11
Table 12	Peak no. 12

Figure 88 Mass spectrum of identified (*Z*)-sabinene hydrate

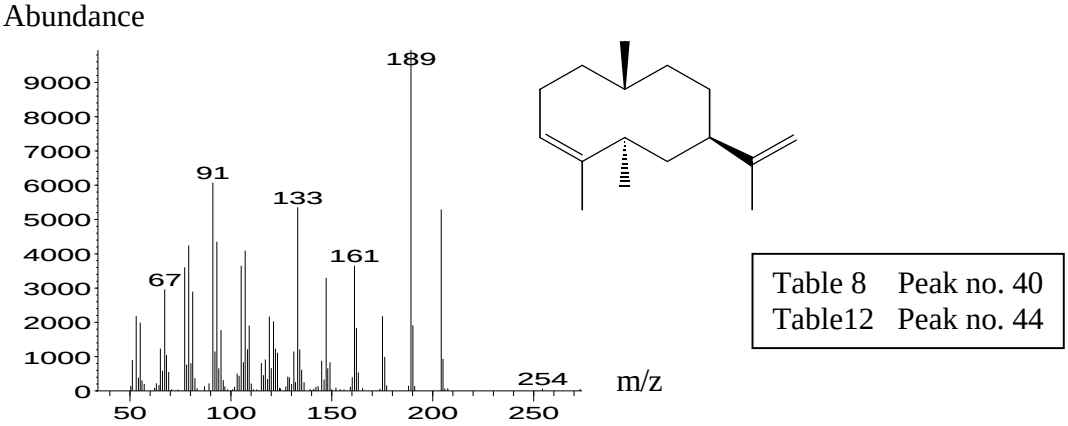


Figure 89 Mass spectrum of identified α -(-)-selinene

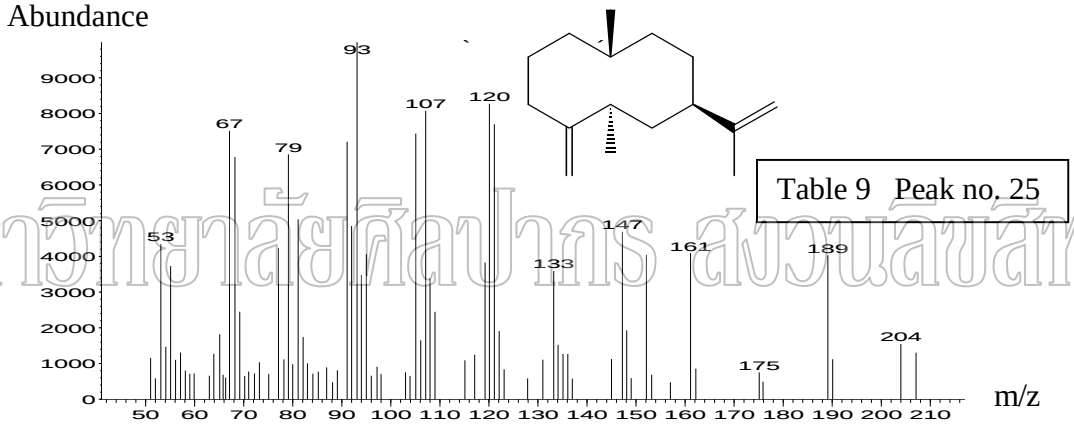


Figure 90 Mass spectrum of identified β-(+)-selinene

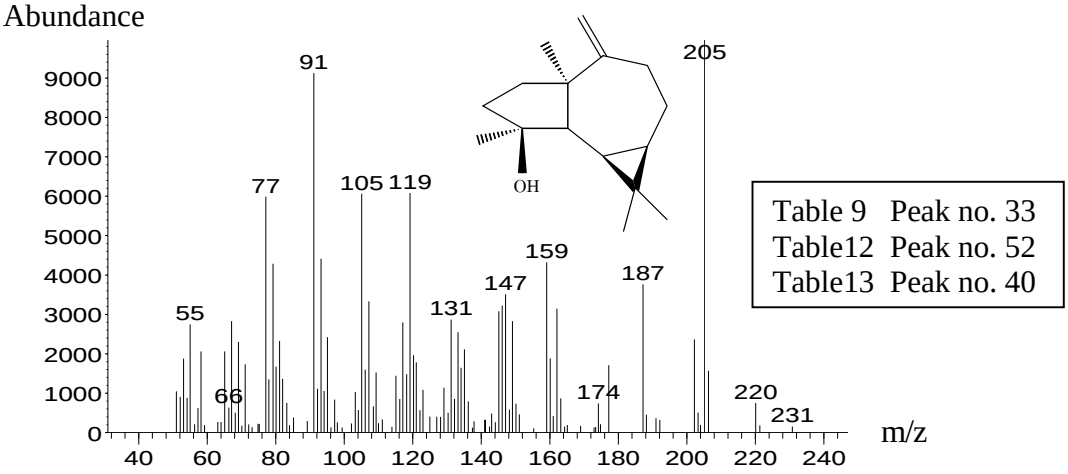


Figure 91 Mass spectrum of identified (+)-spathulenol

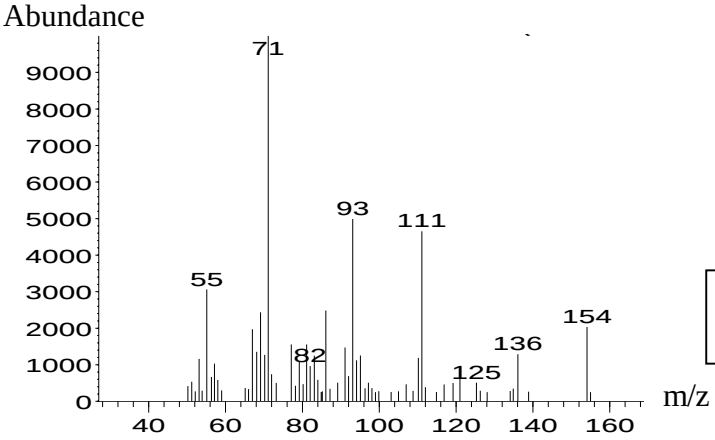


Table 9 Peak no. 17
Table13 Peak no. 20

Figure 92 Mass spectrum of identified terpinen-4-ol

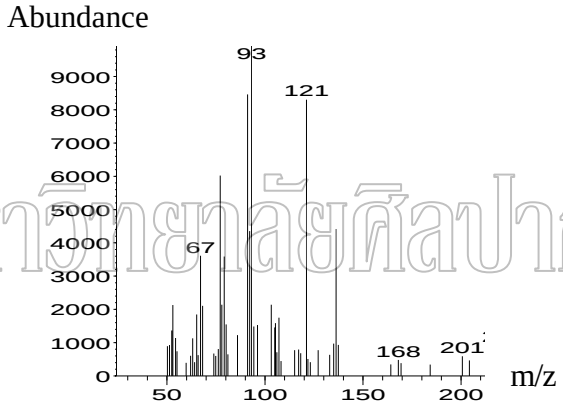


Table 8 Peak no. 10
Table12 Peak no. 11

Figure 93 Mass spectrum of identified γ –terpinene

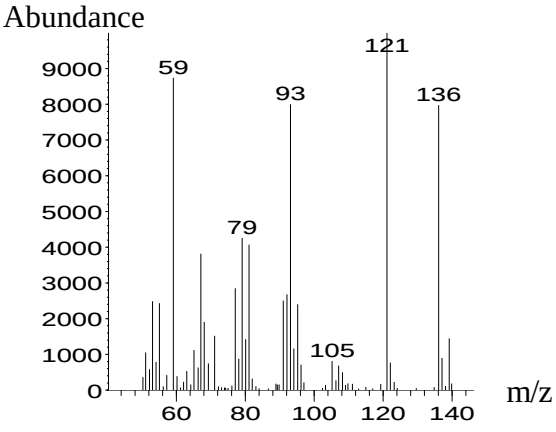


Table 8 Peak no. 17
Table 9 Peak no. 21
Table12 Peak no. 21

Figure 94 Mass spectrum of identified α –terpineol

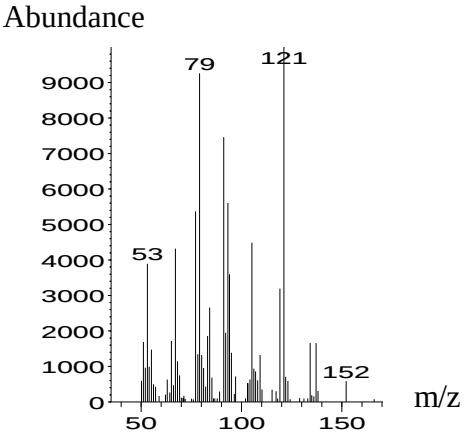


Table 8 Peak no. 18

Figure 95 Mass spectrum of identified γ –terpineol

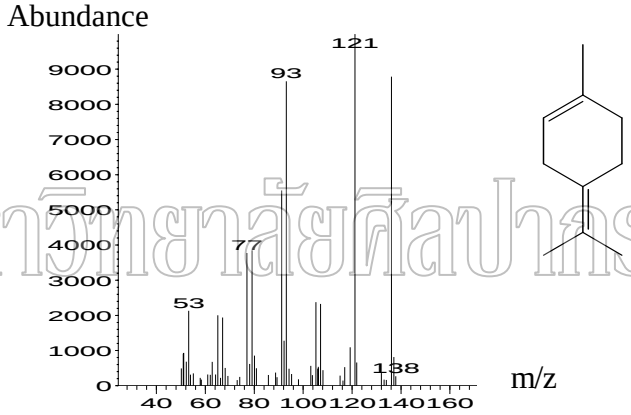


Table 8 Peak no.12
Table 9 Peak no.11
Table12 Peak no.14
Table13 Peak no.11

Figure 96 Mass spectrum of identified terpinolene

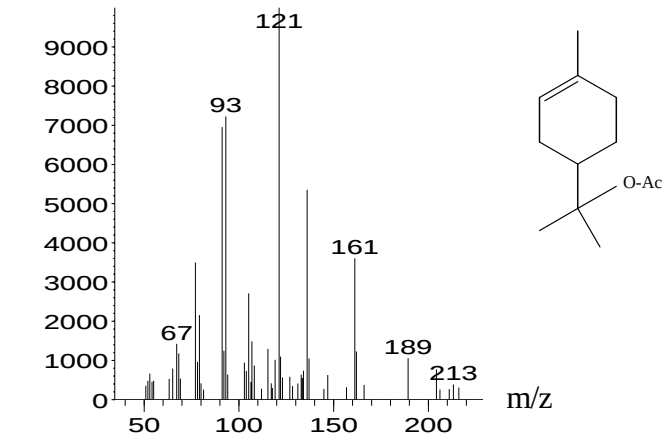


Table 8 Peak no.29
Table12 Peak no.32

Figure 97 Mass spectrum of identified α –terpinyl acetate

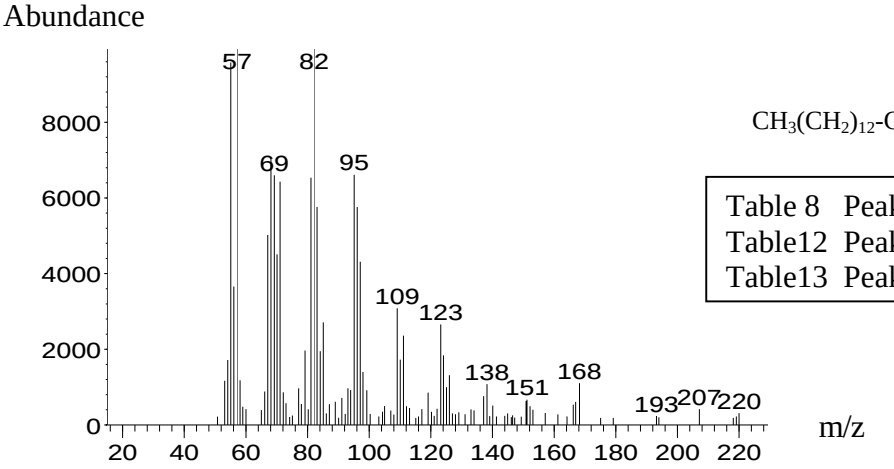
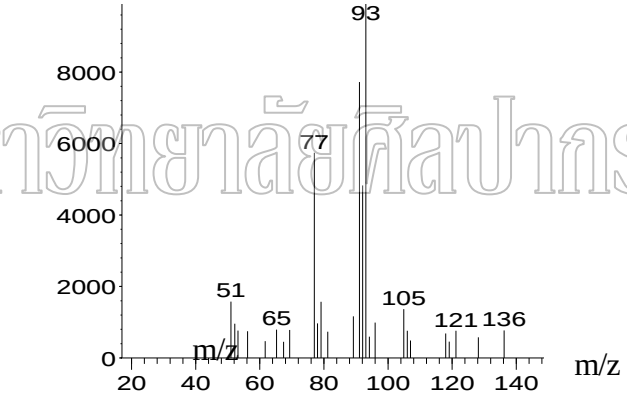


Table 8 Peak no. 47
Table12 Peak no. 54
Table13 Peak no. 37

Figure 98 Mass spectrum of identified tetradecanal



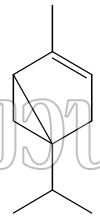
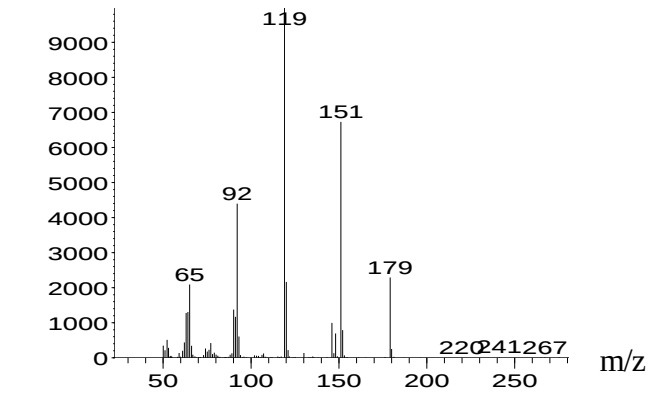


Table 8 Peak no.1
Table 9 Peak no.2
Table12 Peak no.1

Figure 99 Mass spectrum of identified α –thujene



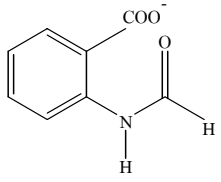


Table12 Peak no. 48

Figure 100 Mass spectrum of identified 2-(formylamino)-benzoate

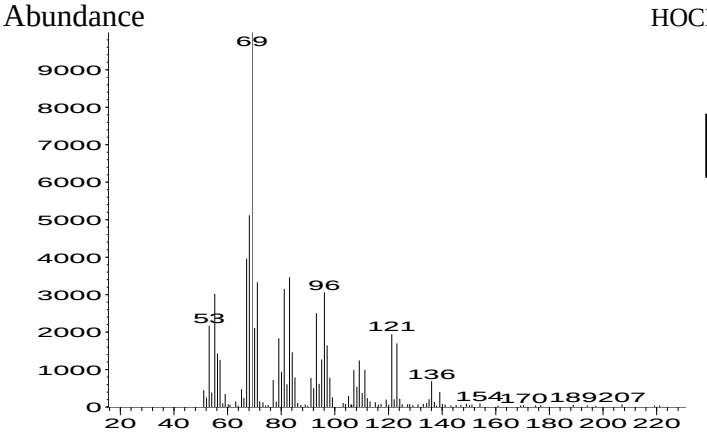


Table 13 Peak no. 36

Figure 101 Mass spectrum of identified 3,7-dimethyl-2,6-octadien-1-ol

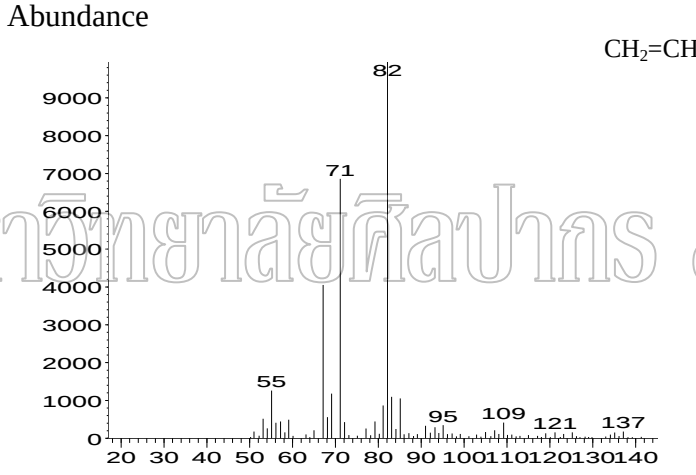


Table13 Peak no. 38

Figure 102 Mass spectrum of identified 3,7-dimethyl-1,5-octadien-3,7-diol

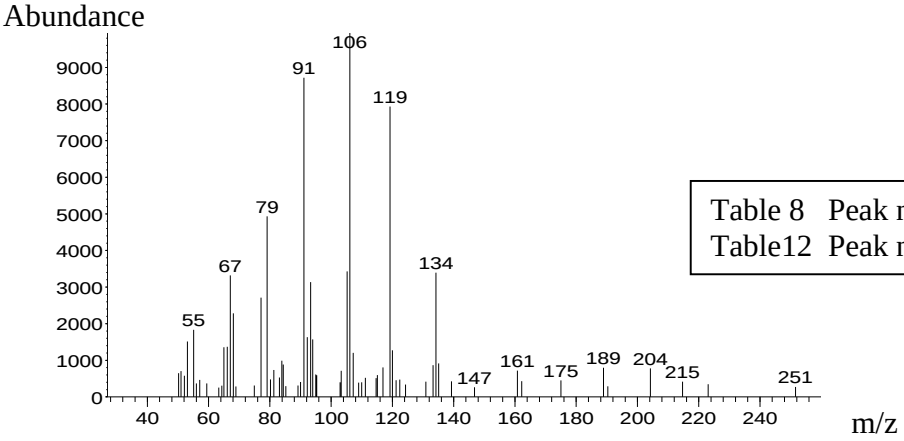


Table 8 Peak no.35
Table12 Peak no.39

Figure 103 Mass spectrum of identified unknown from lime oil

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